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Evaluation of Methods for Genetic Analysis of *Streptomyces scabies*

by

Kimberly Anne Irving ©

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Abstract

Common scab of potato, caused by *Streptomyces scabies*, is the fourth most important disease of potato in North America. By understanding the genetic mechanisms of pathogenicity, novel methods of control may be developed. This study endeavoured to develop methods for genetic analysis of *S. scabies*, which circumvent the putative endogenous restriction system. To facilitate insertional mutagenesis, the efficiency of polyethylene glycol-mediated transformation, electroporation, and conjugation for introducing DNA into *Streptomyces* spp. was evaluated. Exconjugants of S. *scabies* were obtained with plasmid pSET152, this is the first report of conjugative introduction of DNA into *S. scabies.* For identification of known genes, Southern hybridization using a probe constructed from the *hrp* cluster of *Pseudomonas syringae* pv. *phaseolicola* and PCR amplification of an *arpA* homologue were performed. No homologous genes were found in *S. scabies.* These results will provide an opportunity for further genetic analysis in *S. scabies.*

This thesis is dedicated to my family; Mom, Dad and Kelsey.
Their unyielding support, love and belief in me helped me through the rough times, and enhanced the good.

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List of Abbreviations

a	alpha
aacC4	aminoglycoside-3-acetyltransferase IV from pWP7b
AFLP	amplified fragment length polymorphism
AHL	N-acyl-homoserine lactone
amp	ampicillin
apr	apramycin
bp	base pair
C	cytosine
°C	degree Celsius
cam	chloramphenicol
cm	centimetre
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide-5'-triphosphate
EDTA	ethylenediamine tetraacetic acid
g	gram
G	guanine
gen	geneticin
h	hour
HR	hypersensitive response
$int^{F\,C31}$	site specific integration gene from bacteriophage *F* C31
IS	insertion sequence
kb	kilobase
kan	kanamycin
kV	kilovolt
L	litre
LA	Luria-Bertani media with addition of agar
LB	Luria-Bertani liquid media
MCS	multiple cloning site
merp	mercury resistance promoter
mg	milligram
mL	millilitre

mm	millimetre
mM	millimolar
MS	mannitol soya flour medium
^{32}P	phosphorus 32
PAGE	polyacrylamide gel electrophoresis
PAIS	pathogenicity islands
PCR	polymerase chain reaction
PEG	polyethylene glycol
RAPD	randomly amplified polymorphic DNA
rep	replication protein
rep$^{\text{pUC}}$	*Escherichia coli ori* from pUC plasmid
rep$^{\text{SCP2*}}$	streptomycete *ori* from SCP2* plasmid
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SSC	150 mM NaCl, 15 mM sodium citrate
TAE	tris acetate
TBE	tris borate
TE	tris-EDTA
tnpA	transposase
tsr	thiostrepton
U	units
µL	microlitre
YEME	yeast extract-malt extract medium
w/v	weight per volume
v/v	volume per volume

Chapter 1 Literature Review

1.1 Characteristics of *Streptomyces*

1.1.1 *Streptomyces* spp.

Streptomycetes are difficult to classify; they are Gram-positive bacteria that possess many fungal characteristics. Every species gives rise to a differentiated, filamentous mycelium. The vegetative hyphae are slender (about 1μm wide), and branched. During its growth, cell division is infrequent; as a result, hyphal cells are long and contain multiple copies of the genome (Flärdh *et al.*, 2000). Both the extensively branched mycelium and the release of hydrolytic enzymes allow for utilization of complex carbon and nitrogen sources found in the soil (egg. lignins, xylans, cellulose, and chitin) (Hale *et al.*, 1995).

In response to an as yet undetermined signal, aerial hyphae arise from the main hyphae and develop specialized sporulation septa. The signal that initiates the metabolic change may be a nutritional deficiency (Elliot *et al.*, 2001). Unlike vegetative septa, the morphologically distinct sporulation septa are regularly spaced and divide the aerial hyphae into unigenomic spores (Flärdh *et al.*, 2000). The spore chains may be straight, wavy, branched, or spiralled. The morphological features of the aerial mycelium including mode of branching, spore chain configuration, and spore surface are all important in taxonomic identification (Anderson and Wellington, 2001). During spore maturation, the cell wall thickens and pigment is deposited. The non-motile spores are then released from the tips of the aerial hyphae and are dispersed by soil, water, wind, and infected plant residue. The ability to produce spores facilitates survival over long periods of drought, water saturation and frost (Katsifas *et al.*, 2000).

Most *Streptomyces* spp. are soil saprophytes that produce biologically active secondary metabolites, including antibiotics (Bukhalid and Loria, 1997). Approximately 2700 (70%) of the known antibiotics are produced by streptomycetes (Taddei *et al.*, 1999). Secondary metabolism is initiated when the aerial hyphae first form (Elliot *et al.*, 2001), although the particular growth phase is dependent on the nutritional environment (carbon-energy, nitrogen and phosphorus nutrients, and trace elements) in which the organism is grown (Horinouchi and Beppu, 1992; Obanye *et al.*, 1996). Initiation of sporulation and production of antibiotics are dependent on the production of diffusible low-molecular-weight substances called butyrolactone autoregulators. The butyrolactone signalling system is found in many *Streptomyces* spp. (Kudo *et al.*, 1995; Onaka *et al.*, 1998, Kawachi *et al.*, 2000). Production of secondary compounds probably increases the fitness of the species in the highly competitive soil habitat. Biological and industrial interests in Streptomycetes are rapidly increasing due to the vast array of pharmaceutically important antibiotics produced by this genus. It is important that research continues in this field as a detailed understanding of the signalling system, and the genetic components involved in antibiotic production, will allow manipulation of antibiotic production in *Streptomyces* strains (Lee *et al.*, 1999). Commercially, control of antibiotic synthesis would be a tremendous advantage.

Only a small proportion of the close to 500 described *Streptomyces* species are pathogenic. Among *Streptomyces* spp. are known plant pathogens that infect a wide range of crop species ranging from peanuts to potatoes. Plant infection is limited to the underground structures of hosts, with necrosis often the first symptom manifested. Systemic plant infection is not known, although aerial plant structures may be stunted or wilted if root infection is severe.

1.1.2 *Streptomyces scabies*

The most important Streptomycete plant pathogen is *S. scabies* (Thaxter) Lambert and Loria. Originally, it was considered to be a fungal pathogen and was classified as *Oospora scabies* Thaxter by Thaxter in 1891. The pathogenic strains were defined by the production of soluble brown melanin pigment, and grey spores borne in spiral chains. The current description is consistent with the first characterization, and identifies *S. scabies* as a bacterium that produce melanin and loose spiral chains of smooth grey spores (Lambert and Loria, 1989b). As well, *S. scabies* utilizes a group of eight diagnostic sugars (L-arabinose, D-fructose, D-glucose, D-mannitol, rhamnose, sucrose, D-xylose, and raffinose) as their sole carbon source, are sensitive to streptomycin, and do not grow below pH 4.5 (Goyer and Beaulieu, 1997). Based on these phenotypic features, *S. scabies* strains form a homogenous group distinct from other *Streptomyces* spp.

However, DNA-DNA hybridizations (Bouchek-Mechiche *et al.*, 2000) and analysis of 16s rRNA gene sequences (Takeuchi *et al.*, 1996; Kreuze *et al.*, 1999) have shown great genetic diversity between the strains identified as *S. scabies.* The degree of DNA homology between the pathogenic *S. scabies* strains ranges from as low as 20% and up to 70% (Healy and Lambert, 1991; Doering-Saad *et al.*, 1992). A low genetic homology may imply that mobilized pathogenicity genes have been taken up by the different *Streptomyces* spp. found in the immediate environment (Raghava *et al.*, 2000).

Traditionally, identification of *S. scabies* colonies has relied on phenotypic characteristics, such as morphology of the aerial mycelium and spiral spore chains (Faucher *et al.*, 1992). Production of melanin has also been used as a diagnostic characteristic of *S. scabies* (Conn *et al.*, 1998). However, these methods are unable to differentiate between pathogenic and non-pathogenic strains. Keinath and Loria (1998) found that only 3.3% of isolated *S. scabies* strains were pathogenic on potato tubers. The limitations of these diagnostic methods have prompted a search to identify genetic markers for rapid identification of pathogenic *S. scabies* strains. Genetic techniques such as restriction fragment length polymorphisms (RFLPs), analysis of 16S rRNA sequences, and DNA-DNA hybridizations, have been employed. However, the genetic instability and the high degree of polymorphisms between *S. scabies* strains also reduce the efficiency of these techniques for identification of pathogenic strains. It is therefore believed that identification of pathogenicity factors, such as thaxtomin, are the most probable means by which to quantify pathogenic *S. scabies* in soil (Conn *et al.*, 1998).

Streptomyces scabies is a soil- or tuber-borne pathogen. Earlier studies describe soil inoculum as the major determinant for disease development (Lapwood, 1972; Adams and Hide, 1981; Pavlista, 1996). These results are supported by the fact that *S. scabies* inoculum is actually ubiquitous in potato fields. However, Wilson *et al.* (1999) found that seed tuber inoculum was as significant in scab development as soil inoculum. The relative importance of each pathogen source is dependent on the different soil type, pathogen population, environmental conditions, and agronomic practices of the particular location. *Streptomyces* spp. population densities increase in the soil, the rhizosphere, and on tuber surfaces throughout the growing season. The population densities of total actinomycetes in the soil during the early growing season are a useful measure of disease severity (Keinath and Loria, 1989).

The scab pathogen is adapted to well-drained, neutral pH soils (pH 5.2-8.0), where root crops are usually grown. The optimum temperature for growth is 30°C (Loria *et al.*, 1997). Different complexes of *S. scabies* have been identified in areas with non-alkaline soil. Although these species are capable of causing scab, their morphological and genetic characteristics place them in different taxonomic groups. For example, areas of North America with acidic soils are infected with *S. acidiscabies* Lambert and Loria, the causative agent of deep-pitted scab (Lambert and Loria, 1989a). Other species correlated with common scab include *S. turgidiscabies* Tanaka, Takeuchi, Tanii, Miyajima, Abe, and Kuninaga found in Japan (Miyajima *et al.*, 1998), and *S. caviscabies* Goyer, Faucher and Beaulieu (Goyer *et al.*, 1996).

1.2 Common Scab

1.2.1 Epidemiology

Common scab does not have a great impact on tuber yield, but because of the external lesions it reduces the marketability of all grades of potato tubers. Due to the negative aesthetic appeal, common scab is considered to be the fourth most important disease of potato in North America (Loria *et al.*, 1997). This impacts the fresh table and processing industries. Seed potatoes are also negatively affected, as there is the potential to propagate the disease through planting of infected seed. In addition to infecting potatoes, *S. scabies* also causes scab disease on taproots of other crops (Goyer and Beaulieu, 1997). Although vegetables such as radish, turnip, beet, and carrot are infected, the symptoms are minor when compared to those on potato.

The disease cycle begins as the *S. scabies* spores germinate, giving rise to one or two germ tubes. The germ tubes infect tubers by penetrating through developing lenticels in young tubers, and wounds or stomata in older tubers. Because of the preference for invasion through immature lenticels, tubers are most susceptible to infection during the period of tuberization and initial tuber expansion, and for approximately an additional 6-8 weeks (Krištufek *et al.*, 2000). After penetration, the hyphae grow between or through a few layers of peridermal cells. The infected cells die and the pathogen derives nourishment from them. A defence response is initiated in the tuber whereby the surrounding healthy cells rapidly divide and lay down several layers of cork cells that effectively isolate the pathogen (Agrios, 1997). The dead cells of the restricting cork layer are eventually invaded and another cork layer forms below. As the pathogen progresses inwards, hypertrophy of the successively deeper cell layers pushes the cork layers outward. Collapse and shedding of these layers of cork and dead cells creates the symptomatic lesions.

The lesions are most commonly erumpent, but may also appear superficial or pitted. The majority of lesions are 3 to 4 mm deep, however, the depth is dependent on the host variety, soil conditions, virulence of the invading pathogen, and secondary infections through the lesions. For example, insects break down the cork layers, allowing the pathogen to invade deeper into the tuber. The more virulent *S. scabies* strains result in deeper pitted symptoms, due to a high level of thaxtomin production, as discussed later (Loria *et al.*, 1997).

1.2.2 Host Defence

Plants are under continual attack by pathogenic organisms and therefore have developed intricate defence mechanisms to ward off disease. In response, the attacking pathogens have developed equally elaborate methods to induce disease in susceptible host plants. The gene-for-gene hypothesis is the underlying theory establishing the complex relationship between pathogen and host. The gene-for-gene model involves recognition of pathogen released dominant avirulence (Avr) factors by the plant resistance (R) proteins (Flor, 1955). In non-host plants, or those containing the corresponding dominant R gene, this interaction results in development of the hypersensitive response (HR), a localized rapid death of host tissue at the point of infection (Alfano and Collmer, 1997; Sessa *et al.*, 2000). Such a response is seen in the tuber cells infected with, and surrounding, the invading *S. scabies* hyphae. The hypersensitive response is probably the most important and common biochemical defence response a plant host is capable of inducing; it effectively isolates the pathogen from surrounding non-infected tissue, by creating a barrier of dead tissue around the invading hyphae (Shapiro, 2000). The infected tuber, however, further protects itself by laying down the protective cork barrier below the invading hyphae.

1.2.3 Control Strategies

The control of tuber-borne diseases, including common scab, has historically relied on seed certification, tissue culture, crop rotation, limiting the number of generations per field (De Boer, 1994), and application of seed treatments such as Fluazinam (Wilson *et al.*, 1999). All of these control strategies are focused on planting healthy, pathogen-free seed. However, these methods alone will not ensure a disease-free crop as disease escape may occur if asymptomatic infections are present, such as late season infection or contamination of resistant cultivars. There is also the possibility of additional sources of inoculum. Therefore, manipulation of soil pH and moisture level are also prospective avenues for achieving control. Maintenance of high soil moisture during initial tuber development has reduced the severity of scab infection (Loria *et al.*, 1997). Rotating out of taproot crops is one of the easiest control strategies to implement, although it may not be feasible for seed producers or commercial farmers.

Control strategies involving application of organic amendments such as animal manures and composts have produced mixed results. Early studies, showed an increased incidence in scab after the application of animal manures (Blodgett, 1940), and others found that none of the chemical treatments were successful at reducing the level of common scab (Platt and MacLean, 1997). Challenging these results, Conn and Lazarovits (1999) showed that chicken and swine manure were successful at reducing scab infection. Infection control could be the result of increased soil pH, ammonia and nitrate accumulations, or stimulation of competing microbial populations in the soil (Lazarovits, 2001). Even though results from the first year were positive, the authors also noted that three years after treatment, scab infection was twice that of the control. A study by Mizuno *et al.* (2000) found that a single application of ammonium sulfate was successful at lowering soil pH, resulting in suppression of common scab due to an increase in the concentration of water-soluble aluminum. This has led to the belief that it is the uptake of soluble aluminum by the potato plant that is responsible for suppressing scab development, rather than the soil pH as previously believed.

Although these methods have generally proven effective as control measures, they are not always practical. For example, maintaining high soil moisture for an extended period creates an environment that is conducive to the growth of other pathogens, such as *Rhizoctonia solani* Kühn causing black scurf (Errampalli and Johnston, 2001) and *Erwinia carotovora* subsp. *atroseptica* Dye causing blackleg. Soils with pH levels that inhibit *S. scabies* activity are also unfavourable to the growth of many vegetable crops. In addition, the efficacy of these management strategies is dependent on environmental and soil-type differences (Hosaka *et al.*, 2000).

There are also novel control strategies that exploit naturally occurring antimicrobial substances, found in the roots of certain crops and vegetables. For example, *Geranium pratense* root applied as a powder to the soil decreases the tuber area covered with scab lesions (Ushiki *et al.*, 1998).

Biological control of common scab may also be achieved through the use of non-pathogenic *Streptomyces* strains that are antagonistic to pathogenic *S. scabies.* There is strong evidence for the suppression of potato scab in the field by non-pathogenic strains of *Streptomyces* (McQueen *et al.*, 1985; Emmert and Handelsman, 1999; Schottel *et al.*, 2000). Antagonistic strains were first isolated from fields that had been used in potato monoculture for 23 years (Lorang *et al.*, 1989). When strains of *Streptomyces* were isolated from the lenticels of tubers harvested from the disease suppressive fields, they were found to be suppressive to pathogenic *Streptomyces* strains (Lorang *et al.*, 1995). The identified strains reduced the incidence and severity of scab development and had no negative impact on tuber yield (Liu *et al.*, 1995). Suppression of pathogenic strains by non-pathogenic strains could be a case of simple competitive exclusion, with the suppressive strain more easily and rapidly colonizing the tuber lenticels, in effect preventing the pathogen from entering. It is also believed that antibiotic inhibition of the pathogenic strains (Neeno-Eckwall and Schottel, 2000; Neeno-Eckwall *et al.*, 2001) and interspecies communication (Becker *et al.*, 1997) are involved in disease control.

Although there are relatively few studies that show a direct link between cell density and regulation of *Streptomyces* genes through autoinducers, communication is known to be important in antibiotic synthesis. Sanchez and Brana (1996) showed that an accumulation of an extracellular conditioning factor in a cell-density dependent manner was sufficient to initiate antibiotic synthesis in *S. clavuligerus*. Interspecific communication between other *Streptomyces* spp. has also been noted (el-Abyad *et al.*, 1993). If there is a more comprehensive understanding of the antibiotic production system and its dependence on cell density-dependent communication, it may be possible to select for strains with enhanced levels of suppression to *S. scabies*.

Unfortunately, there are very few highly resistant potato cultivars and no immune cultivars. Known scab resistant cultivars include Russet Burbank and Superior (Loria *et al.*, 1997). Recently, three wild ancestors of cultivated resistant species have been identified as scab resistant (Hosaka *et al.*, 2000). These wild species could potentially be used as primary gene sources for scab resistance. Genetic screening of cultivated potato species is complicated due to their tetraploid chromosome number and genetically heterozygous nature of all varieties (Tai, 1998). However, the use of marker-assisted selection (RFLP, AFLP, RAPD) and genetic transformation should facilitate the search for, and development of, resistance in common potato species (van der Voort *et al.*, 1997).

1.3 Pathogenicity

1.3.1 Thaxtomins

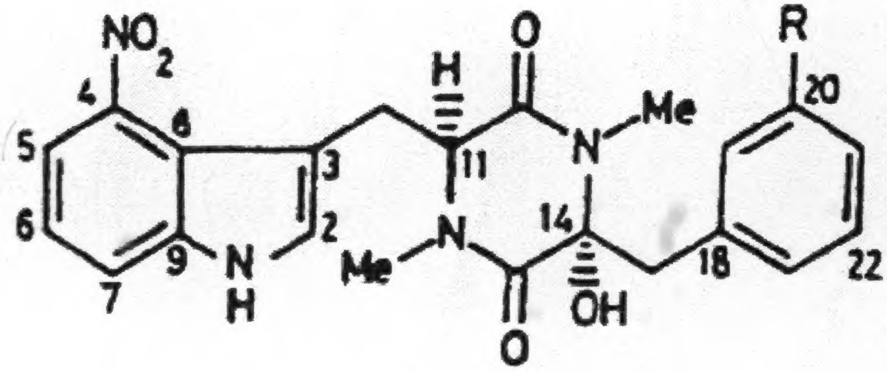
Although there is speculation about the mechanism of pathogenicity of *S. scabies*, it is known that the organism secretes extracellular hydrolytic enzymes and phytotoxins to gain access to the tuber tissue (Fellows, 1926; Jones, 1931; Sakai *et al.*, 1984). A proposed mechanism of pathogenicity is the production of thaxtomins by certain *Streptomyces* spp. Originally purified from *S. scabies*, these toxins belong to a family of unique phytotoxic secondary metabolites (King *et al.*, 1989). Structurally, thaxtomins are 4-nitroindol-3-yl-containing 2,5-dioxopiperazines (Figure 1-1). Of the 11 different thaxtomins produced by plant-pathogenic *Streptomyces* sp., thaxtomin A is the most predominant (Loria *et al.*, 1997).

Goyer *et al.* (1998) showed that *S. scabies* mutants unable to produce thaxtomin A were rendered non-pathogenic. Tuber symptoms produced by *S. scabies* and those produced by application of purified thaxtomin are similar; as a result, thaxtomin production is positively correlated with pathogenicity and degree of virulence. In a series of surveys extending throughout North America, Europe and Australia, a significant number of pathogenic *Streptomyces* isolates from scab-infected potato tubers were associated with thaxtomin production (King *et al.*, 1991; King *et al.*, 1992; King and Lawrence, 1996; el Sayed, 2000). The discovery that thaxtomin A is produced by unrelated pathogenic *Streptomyces* spp. and is not produced by non-pathogens, has strengthened this correlation. These findings imply that thaxtomin production will be a useful plant pathogenicity determinant for *S. scabies*.

There is an important role of thaxtomins in *S. scabies* pathogenicity, although the exact nature of interaction between tuber cells and thaxtomins has not yet been determined. Electron microscopy studies show that thaxtomin application causes cell collapse in potato tubers (Stein *et al.*, 1995), cell hypertrophy at low doses, and is lethal at doses equal to those in *S. scabies* infections (Lawrence *et al.*, 1990). This toxin also has a general deleterious action on the plasma membrane of those cells not collapsed (Goyer *et al.*, 2000). While evaluating the herbicidal potential of thaxtomins, King and Lawrence (2001) found that the phytotoxicity of thaxtomin A and its analogues was partly due to inhibition of cellulose biosynthesis in plant cells. All of these effects would contribute to infection in the dead tissue.

During the search for a genetic determinant of thaxtomin production, the *nec1* gene was identified when a cosmid library of *S. scabies* was expressed in *S. lividans* Shirling and Gottlieb (Bukhalid and Loria, 1997). Although expression of *nec1* in *S. lividans* failed to induce production of the previously described thaxtomins, the strain did produce an extracellular water-soluble compound that was sufficient to cause necrosis of potato disks. Southern hybridization revealed that *nec1* is homologous to genes in other

Figure 1-1. Chemical structure of thaxtomin A and B (King *et al.*, 1991).


NO2
4
5
6
7
8
9
N
H
3
2
H
11
O
N
Me
Me
N
14
O
OH
R
20
18
22

(A) R = OH

(B) R = H

Streptomyces strains that produce thaxtomin, but is absent from non-pathogenic strains that do not produce thaxtomin. These results indicate a role for *nec1* in pathogenicity, even though it is not the main thaxtomin biosynthetic gene.

Sequencing of the regions flanking *nec1* identified a neighbouring open reading frame (ORF$_{tnp}$), with similarity to a family of transposases that are conserved among unrelated plant pathogens and absent from non-pathogens (Healy *et al.*, 1999). This family of transposases is known to mobilize antibiotic resistance genes among various bacterial species. The presence of this transposase in the vicinity of a pathogenic gene suggests that the ORF$_{tnp}$-*nec1* complex has been horizontally transferred between pathogens (Bukhalid *et al.*, 1998). This is supported by the low GC content (54%) of *nec1* when compared to the coding regions characteristic of the *Streptomyces* genome, the production of thaxtomin in unrelated species, and conservation of the ORF$_{tnp}$-*nec1* sequence among genetically diverse *Streptomyces* plant pathogens (Bukhalid *et al.*, 2002).

Because pathogenicity factors are usually clustered together, the regions adjacent to *nec1* were examined for the presence of other pathogenic genes. Sequencing of the regions flanking *nec1* resulted in the identification of a pathogenicity-associated insertion sequence (IS) element, IS1629 (Healy *et al.*, 1999). These IS elements are the simplest of genetic mobile elements. Identification of an IS adjacent to a highly conserved pathogenicity factor has led to the belief that this is one end of an uncharacterized pathogenicity island.

Recently, two other genes involved in thaxtomin production have been identified in *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies* (Healy *et al.*, 2000). These genes, *txtA* and *txtB*, code for putative peptide synthetases involved in thaxtomin production. Based on their structure, Healy *et al.* (2000) predicted that thaxtomins are synthesized by peptide synthetases. Degenerate primers from the A2 and A3 conserved domains of known peptide synthetases were used in PCR amplification from three *Streptomyces* spp. (*S. scabies*, *S. acidiscabies*, and *S. turgidiscabies*). The PCR products were used to probe genomic digests of the three species, and identify a conserved peptide synthetase locus. Sequence and mutational analysis of the two cloned genes corroborate the role of *txtAB* genes as thaxtomin peptide synthetases.

1.3.2 Esterase

Suberin is a waxy polyester compound composed of long chain fatty acids and phenolics, normally produced by plants to protect against desiccation and possibly pathogen invasion. A pathogen may gain entry into the plant by secreting enzymes that break down the protective suberin that proliferates at the site of infection (Babcock *et al.*, 1992). Unlike thaxtomin production, esterase activity is not unique to pathogenic *Streptomyces* strains. For example, a pathogenic strain of *S. acidiscabies* and a non-pathogenic strain of *S. badius* Pridham. both produce esterase when grown on a cutin substrate (Goyer *et al.*, 1996). The ability to break down suberin is also beneficial to saprophytes in nutrient acquisition.

The first esterase to be isolated from an *S. scabies* strain used PNB (*p*-nitrophenyl butyrate) as its substrate (Lin and Kolattukudy, 1980). McQueen and Schottel (1987) isolated and characterized a novel extracellular esterase (FL1) produced by two different pathogenic strains of *S. scabies*. The differences in heat stability, substrate saturation, and molecular weight indicated that the two esterases are unique. Although it was not proven that suberin is a substrate for the second enzyme, its localization in two different pathogenic strains, its absence in the non-pathogenic strains, and its activation by suberin, all suggest a role in pathogenicity. The esterase gene (*est*) from *S. scabies*, responsible for encoding the FL1 esterase, was later cloned and sequenced (Raymer *et al.*, 1990).

To further characterize *est*, it was expressed in *S. lividans* and *Escherichia coli* Migula (Hale *et al.*, 1992; Schottel *et al.*, 1992). Features of the expressed protein, such as timing of expression, processing site for signal peptide cleavage, and the secretion pathway were similar to those produced in *S. scabies*. Furthermore, deletion analysis indicated that the hydrophobic domain of the signal peptide is required for secretion, and the presence of a specific 23-bp protein-binding site upstream of the coding sequence is important in *est* gene expression. It is hypothesized that a putative regulatory protein binding to this site functions as a positive transcription regulator (Babcock *et al.*, 1992).

1.4 Barriers to Progress in *Streptomyces* spp. Genetic Research

To date, only six genes have been identified in *S. scabies* (Raymer *et al.*, 1990, Hale *et al.*, 1995; Bukhalid and Loria, 1997; Bukhalid *et al.*, 1998; Healy *et al.*, 1999; Healy *et al.*, 2000), and very few *Streptomyces* species have been completely characterized. The main factors hindering genetic analysis of Streptomycetes are the lack of natural competence for DNA uptake, a lack of suitable vectors (Burke *et al.*, 2001), the wide diversity of species within the genus (Weaden and Dyson, 1998), and the presence of specific type II restriction modification systems in many *Streptomyces* strains (Chater *et al.*, 1982). The spontaneous genetic instability, megabase deletions, and high-copy number DNA amplifications (Volff and Altenbuchner, 1997) in *Streptomyces* spp. may also be limiting factors in vector transformation.

The high G+C content of the *Streptomyces* linear genome, estimated to be 74% (Wright and Bibb, 1992), reduces the frequency of restriction enzyme target sites that are high in adenine and thymine residues, such as *Eco*RI, *Hin*dIII, and *Hpa*I (Chater *et al.*, 1982). As a result, the choice of vectors available for transfer into *Streptomyces* spp. is limited. In order to circumvent the lack of suitable vectors for genetic analysis, heterologous probes are often used for gene identification. The *secY* gene codes for an integral transmembrane component of prokaryotic protein translocases, which comprise part of the general secretory pathway (Akiyama and Ito, 1987). Identification of a *secY* homolog from *S. scabies* relied on degenerate oligonucleotide probes designed from the conserved region of aligned *secY* genes (Hale *et al.*, 1995). Even though the use of degenerate probes with a G/C bias in the third position of a codon (Wright and Bibb, 1992) increases the likelihood of amplification and subsequent identification, there are still limitations. The genetic diversity within *Streptomyces* spp. and the requirement for at least 60% homology between probe and target sequence, hamper the search for homologous sequences.

The specific restriction system that many strains possess also limits the opportunities for cloning in *Streptomyces*, as there is enzymatic digestion of the foreign DNA before stable transformation occurs (Matsushima *et al.*, 1987). There have been many attempts to bypass the restriction modification systems. Incorporation of self-DNA, transformation with DNA propagated in a non-methylating *Streptomyces* host, or use of a non-methylating strain of *Escherichia coli* as a host (Kieser *et al.*, 2000) are methods which have been attempted. Amplification by PCR also generates non-methylated DNA that is suitable for most transformations. Although most of the known restriction systems ignore ssDNA, the methyl-sensing restriction system of *Streptomyces* is not avoided when ssDNA is used in transformations (Oh and Chater, 1997), further restricting the possibilities for genetic analysis.

1.5 Methods of Genetic Analysis of *Streptomyces* spp.

1.5.1 Southern Hybridization

Southern hybridization analysis is dependent on a labelled single-stranded DNA or RNA sequence (probe) recognizing and binding to a complementary sequence (target) that is immobilized on a solid support. The strength of hybridization is dependent on a number of factors, including the extent of base-pairing that occurs between probe and target. Unfortunately, the degree of mispairing, even under the least stringent conditions, may only be a minimum of 40% to ensure stable hybridization (Cornish *et al.*, 1998). This is the most problematic feature of Southern hybridization, when used in *Streptomyces* genetic analysis. The lack of significant genetic homology between the *Streptomyces* spp. negatively influences the probability of strong probe hybridization, and makes gene detection through this method difficult. Despite these difficulties, various studies using Southern hybridization with *Streptomyces* have been successful in gene identification (Yao and Vining, 1994; Healy *et al.*, 2000). As a result, it is possible that this technique can be utilized in genetic analysis of *S. scabies*.

1.5.2 PCR Amplification

The polymerase chain reaction (PCR) is routinely employed in the detection and amplification of specific DNA or RNA sequences (Ostiguy *et* al., 1996; Parro *et al.*, 1999; Johnson, 2000). This technique may be used for bacterial gene identification and cloning, and as such it can substitute for conventional DNA probe-hybridization assays. As compared with probe hybridization, PCR is more rapid and extremely sensitive. This sensitivity allows detection of targeted genes at very low concentrations. However, PCR is less tolerant of low-level sequence degeneracy than is probe hybridization (Goodman, 1995). This is an important factor when analysing *Streptomyces* spp., due to the heterogeneity of their genomes.

1.5.3 Insertional Mutagenesis

Non-specific mutants generated through traditional methods, such as UV irradiation (Anderson, 1995) are undesirable for identification of single genes. Plasmids and transposable elements are able to insert into the genome in a specific, unique location and ensures that any phenotypic change is the result of a single insertion into the targeted sequence.

Genetic competence, the ability of bacteria to take up exogenous DNA, is conserved in many phylogenetically different bacteria (Maas *et al.*, 1998). There are various hypotheses as to why these elaborate systems have developed and been maintained in such a variety of species. The first proposal is that competence evolved to permit uptake of DNA as a food supply (Finkel and Kolter, 2001). The extracellular DNA serves as the sole carbon and energy source, supporting microbial growth. Secondly, some believe that transformation serves in DNA repair (Hoelzer and Michod, 1999). Finally, transformation may be a mechanism for creating genetic diversity by generating new allelic combinations (Dubnau, 1999). This theory is strongly supported by the presence of mobile DNA sequences, transposons and insertion sequences in many members of the genus *Streptomyces* (Solenberg and Burgett, 1989; Rodicio *et al.*, 1992; Chen *et al.*, 1992). Although all of these individual theories are plausible, competence is most likely maintained due to pressure from a combination of the mechanisms.

1.5.3.1 PEG-Mediated Transformation

Traditionally, artificial transformation takes place through polyethylene glycol (PEG)-mediated DNA uptake by protoplasts (Bibb *et al.*, 1978). The specific conditions for protoplast formation, PEG transformation, and protoplast regeneration vary, and must be optimized for each strain used (Ogawa *et al.*, 1983; Jandova and Tichy, 1990; Zhang *et al.*, 1997). For example, *S. virginiae* Grundy is competent in early stationary phase; whereas *S. lividans* gives highest transformation frequencies when the cultures are in late logarithmic growth (Chater *et al.*, 1982). To complicate matters even more, the conditions required to generate highly competent protoplasts are not necessarily those that facilitate good regeneration. The most important parameters for successful transformation are the mycelial growth phase, the temperature of incubation, the number of protoplasts used per transformation, and composition of the regeneration medium (Kieser *et al.*, 2000). When these conditions are optimized, transformation frequencies of 10^6-10^7 transformants per microgram of plasmid DNA can be achieved with *S. lividans* and *S. coelicolor*.

The mechanism by which PEG mediates transformation is not clearly understood. It could make the cell membrane more permeable to DNA, or it could react with the DNA molecules making them more compact and thereby facilitate DNA uptake. Many species of *Streptomyces* are resistant to PEG transformation, mainly due to the presence of restriction systems. Inhibition of the restriction system can sometimes be achieved through heat treatment of the protoplasts prior to transformation. The particular temperature used for heat inactivation is dependent on the strain, as many species are rendered non-viable at high temperatures (Bailey and Winstanley, 1986; this study). However, *Streptomyces* strains may still be resistant to transformation after heat treatment. Because of species specificity, the difficulty in working with fragile protoplasts, the lack of reproducibility and its limited use on an industrial scale, protoplast transformation has largely been replaced by other methods.

1.5.3.2 Conjugation

Natural conjugation is a common event among *Streptomyces* spp. (Hopwood and Merrick, 1977); as a result it can be exploited for *in vitro* transformation. Conjugation between *Streptomyces* and a non-methylating strain of *E. coli* reduces the effects of the restriction system, and allows DNA transfer at fairly high frequencies (Flett *et al.*, 1997; Voeykova *et al.*, 1998). Conjugation is a widespread plasmid- or transposon-encoded process by which DNA is transferred from a bacterial donor to a recipient cell in intimate contact (Krause *et al.*, 2000). Conjugative DNA transfer is classified as type-IV secretion (Salmond, 1994). Type-IV secretion is mediated by multi-protein transmembrane complexes, whose genes are organized in a single cluster. Filamentous appendages, such as pili, are also required (Krause *et al.*, 2000). The transmembrane complex consists of essential transfer (Tra) proteins, which form the conjugative pili responsible for initiation of donor-host interaction (Giebelhaus, *et al.*, 1996).

The popular use of this method stems from its simplicity and high efficiency. Because conjugation involves the experimentally tractable host *E. coli*, DNA transfer is greatly simplified (Flett *et al.*, 1997). An *E. coli* host can be used to replicate the vectors and easily produce and manipulate the desired DNA constructs.

The most beneficial aspect of conjugation is the potential to reduce the effect of the restriction systems possessed by many *Streptomyces* strains, as compared to DNA introduction via other methods (PEG-mediated uptake, electrotransformation). Based on previous findings that indicated a methylation-deficient strain of *E. coli* could effectively introduce foreign DNA into *S. avermitilis* via conjugation, MacNeil *et al.* (1992) constructed the stable methyl-deficient *E. coli* strain ET12567 (*dam*-13::Tn*9*, *dcm*-6, *hsd*M). Even though conjugation with some strains of *Streptomyces* are still subject to restriction, high conjugation frequencies may permit a sufficient number of plasmids to avoid restriction. Conjugative DNA transfer into highly restrictive strains, such as *S.*

fradiae Waksman and Henrici (Bierman *et al.*, 1992) and *Saccharopolyspora spinosa* Mertz and Yao (Matsushima *et al.*, 1994) is considered successful, with transconjugant frequencies of 10^{-1} and 10^{-7}, respectively.

The *E. coli* host must possess certain traits to ensure successful conjugation. Because of the methyl-specific restriction system of *Streptomyces*, it is essential that the host is non-methylating. Generally, the plasmid will contain the *oriT* fragment but the *E. coli* host must provide the transfer function, usually through a non-transmissible driver plasmid. For *Streptomyces* transformations, the most commonly used driver plasmid is pUZ8002. Because of a slightly mutated origin of transfer (*oriT* $^{-}$) (Flett *et al.*, 1997) there is no possibility of the driver/helper plasmid being introduced into the *Streptomyces* host, and therefore no chance for plasmid incompatibility.

1.5.3.3 Electroporation

Electroporation is routinely used for high efficiency transformation of Gram-positive and Gram-negative bacterial species (Dower *et al.*, 1988; Diver *et al.*, 1990; Haynes and Britz, 1990). By subjecting a mixture of competent cells and DNA to a brief, high-voltage electrical pulse, transient membrane pores are created that facilitate uptake of the DNA. Between 10^{9} to 10^{10} transformants/μg of DNA can be obtained through electroporation (Dower *et al.*, 1988). Electroporation has been used for bacterial species that are normally refractory to transformation, including many *Streptomyces* spp. (Pigac and Schrempf, 1995; Mazy-Servais *et al.*, 1997).

An advantage of electroporation is that protoplasts are not required. As a result, electroporation is less tedious and time-consuming than traditional protoplast transformation. However, the conditions for electroporation must be optimized for each strain. Electroporation of mycelia works best on strains that fragment during growth, rather than growing as large mycelial masses in liquid media (Kieser *et al.*, 2000). Prior to electrotransformation, most strains require treatment of the mycelium with low levels of lysozyme (Mazy-Servais *et al.*, 1997) to weaken the mycelial walls and promote pore formation. The most critical parameters for electrotransformation are pre-treatment of mycelium, buffer composition, DNA concentration, and electric field strength.

As with traditional transformation, addition of PEG to the reaction mixture increases efficiency of electroporation (Mahillon *et al.*, 1989). The concentration and source of PEG seem to be important. It is believed that PEG facilitates electroporation through volume exclusion (increasing the effective DNA and cell concentration), interactions with cell membranes, and increased survival of electroporated cells (Pigac and Schrempf, 1995).

1.6 Objectives

The overall objective of this project was to identify and characterize novel genes in *S. scabies*, preferably, the genes involved in pathogenicity determination. However, this was not a strict criterion. The initial course of action was to investigate the utility of transposon mutagenesis, through PEG-mediated transformation of *S. scabies* protoplasts. The transposon vectors were replicated in the permissive, non-pathogenic *S. lividans*, in order to acclimatize the plasmid to *Streptomyces* and thus improve protoplast transformation efficiency. The transformed transposon vector was isolated and introduced into *S. scabies*. Due to the fact that *S. scabies* proved intransigent to protoplast transformation, other avenues were designed to identify *S. scabies* genes.

The specific objectives were:

1. To evaluate the methods of PEG-mediated transformation, electroporation and conjugation for introduction of different plasmid constructs into *S. scabies*.
2. To determine if *hrp* genes could be identified in *S. scabies* by Southern hybridization with *hrp* gene clusters from *Pseudomonas syringae* pv. *phaseolicola*.
3. To determine if an *arpA* homologue could be amplified from *S. scabies* through PCR using degenerate primers.

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Chapter 2 Analysis of Methods for DNA Introduction into *Streptomyces* spp.

2.1 Introduction [1]

Insertional mutagenesis requires the introduction of exogenous DNA into a host's genome. In order to facilitate this, techniques such as electro-transformation, PEG-mediated protoplast transformation and conjugation of *Streptomyces* spp. have been developed. As indicated before, each of these techniques has their limitations, however, after optimization they have all been successfully used to introduce DNA into many species of *Streptomyces.* Introduction of exogenous DNA into *S. scabies* has not been reported.

Generally, the introduced DNA is in the form of a plasmid. The exogenous DNA has specific requirements, depending on the method of introduction. Insertion may be targeted or random, and the plasmids may or may not replicate in the *Streptomyces* host; the choice of which depends on the purpose of the experiment (Bierman *et al*., 1992). Vectors that do not replicate in *Streptomyces*, but integrate into the chromosome, yield stable recombinant strains (Flett *et al*., 1997). This reduces the level of antibiotic selection that is required, and helps circumvent potential plasmid incompatibility arising between resident plasmids and the transformed plasmids. The most useful vectors are capable of replication and selection in both *E. coli* and *Streptomyces* (Larson and Hershberger, 1986). These shuttle plasmids are easily constructed and manipulated in *E. coli* and phenotypic analysis is then done in the test *Streptomyces* spp. If it is important to cure the transformed *Streptomyces* of the delivery plasmid, lack of a partitioning (*par*) function is useful. The bifunctional plasmid pHJL400 (Larson and Hershberger, 1986) lacks a *Streptomyces* partition function, rendering the plasmid segregationally unstable and easily lost (Yang *et al*., 1996), in the absence of antibiotic selection.

Transposable elements are discrete segments of DNA capable of moving from one locus to another in their host genome or between different genomes (Wessler, 1998). The main advantage of transposable elements is their ability to integrate in single copy within the host genome. Because of this specificity, transposable elements, including transposons and IS elements, are important tools for molecular analysis of bacterial genomes (Girard and Freeling, 1999). They are used for such techniques as insertional mutagenesis, adding antibiotic markers or promoters, mapping genetic information, or inserting primer-binding sites (Baltz *et al*., 1997).

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Transposons must have a means of transferring into their target host, this is usually achieved with self-possessed transposase genes. Having a marker, such as antibiotic resistance, temperature-sensitivity (Volff and Altenbuchner, 1997b), or pock-induction (Bibb *et al.*, 1978) greatly facilitates selection of stable transposition events. The choice of antibiotic markers is limited, since many *Streptomyces* strains are naturally resistant to certain antibiotics. For instance, *S. coelicolor* and *S. lividans* are resistant to chloramphenicol and tetracycline, and the majority of ß-lactam antibiotics are ineffective in selection of streptomycetes (Kieser *et al.*, 2000). On the other hand, some strains of *Streptomyces* are susceptible to various antibiotics, even when transformed with a selective plasmid. For example, *S. scabies* shows extreme sensitivity to low levels of thiostrepton and apramycin (this study). It is a relatively easy procedure to test the resistance/selection of a particular antibiotic, and thereby choose an appropriate selective marker.

Various transposons that possess the aforementioned attributes have been developed and are employed for genetic studies of *Streptomyces*. The temperature sensitive transposon delivery plasmid pJOE2577 carrying the Tn*5* derivative Tn*5493* (Volff and Altenbuchner, 1997b) was originally tested with *S. lividans* (Volff and Altenbuchner, 1997a); there is no indication in the literature that this plasmid was used to transform either *S. coelicolor* or *S. scabies*. Other examples of transposons include the integrating antibiotic resistance gene cassettes, Ω*aac*, Ω*hyg*, and Ω*vph* derived from the Ω interposon (Blondelet-Rouault *et al.*, 1997). Although these integrating elements have been used in many *Streptomyces* strains, their use in *S. scabies* has not yet been demonstrated.

Generally, the use of vectors without a transposase gene relies on recombination between the vector insert and its homologue in the host genome. Double crossover events required for gene replacement make up a minority of the total number of transformation events. The more common events are single crossovers that result in integration of both plasmid and insert. In order to detect the infrequent segregants in which a double crossover occurred, extended rounds of growth and replication are required. Some studies have shown that the use of denatured single stranded plasmid DNA leads to an increase in double crossover events (Oh and Chater, 1997).

Due to the ease of its introduction, plasmid pIJ486 is routinely used in *Streptomyces* transformation events as a positive control (Kieser *et al.*, 2000). This plasmid has been used with both *S. lividans* and *S. coelicolor* (Leskiw *et al.*, 1991) but has not been tested in *S. scabies*. Larson and Hershberger (1986) constructed plasmid pHJL400 to be a non-integrating, autonomously replicating plasmid. It was successfully transformed into *S. lividans* but was not tested with either *S. coelicolor* or *S. scabies*.

The most frequently used conjugative vectors are divided into three distinct types: (1) those that integrate into the genome by homologous recombination, (2) those that integrate site-specifically using the bacteriophage F C31 integration functions, and (3) those that do not integrate, but replicate autonomously (Bierman *et al.*, 1992). Despite the obvious differences between vectors, there are certain traits that all conjugative vectors must possess to promote transformation. The most useful vectors contain the origin of transfer (*oriT*) of the IncP-group plasmid RK2/RP4 (Flett *et al.*, 1997). Plasmids that do not replicate in *Streptomyces* are preferred, as there is no chance for plasmid incompatibility. In addition, some autonomously replicating plasmids have been shown to reduce antibiotic biosynthesis (Solenberg *et al.*, 1996). Those plasmids that do not integrate into *Streptomyces* and are non-replicative, are useful as delivery vectors since they are more easily cured. Site specific insertion of vectors derived from bacteriophage A2 is dependent on phage *attP* and bacterial *attB* attachment sites, located within the bacterial genome and a corresponding plasmid-borne integrase gene (Alvarez *et al.*, 1998). Shigeru *et al.* (2000) found that pSET152 plasmid (Bierman *et al.*, 1992) integration occurred at the neutral F C31 *attB* site of *S. lavendulae* FRI-5, and there was no deleterious effect from this insertion. As a result, integrating plasmids of this type, carrying a homologous/mutated DNA insert, may be used for conjugal transformation aimed at targeted gene disruption and mutant complementation studies. Integrating vectors are segregationally stable and therefore must be excised by restriction digestion.

Plasmid size is also important in conjugation. Because *Streptomyces* are generally transformed via rolling circle transformation (Maas *et al.*, 1998), smaller plasmids will transform at higher frequencies. With this method of DNA introduction, larger plasmids have a longer "tail" that must be dragged into the bacteria allowing for a greater chance of error. At 5.5 kb, the plasmid pSET152 is relatively small, and is therefore efficiently transferred (Bierman *et al.*, 1992). Plasmids derived from F C31-containing bacteriophages have slight size limitations on the fragments they can introduce into bacteria. These plasmids cannot accommodate an insert fragment larger than 8 kb, although this is generally a sufficient insert size for most applications. However, other studies have shown that very large plasmids can also be transformed with reasonable frequency. The 48 kb cosmid pRHB304 is transferred by conjugation and forms stable recombinants, by inserting in the F C31 *attB* site in *S. toyocaensis* (Matsushima and Baltz, 1996), or by homologous recombination in *S. spinosa* (Matsushima *et al.*, 1994).

Many conjugative vectors have been developed (Smokvina *et al.*, 1990; Bierman *et al.*, 1992; Motamedi *et al.*, 1995; Voeykova *et al.*, 1998; Fouces *et al.*, 2000). More specifically, the autonomously replicating plasmid pKC1218 (Bierman *et al.*, 1992) has been successfully introduced through conjugation into various *Streptomyces* spp. However, there is no report of its introduction into *S. scabies.* The conjugal integrating plasmid pSET152 is routinely used in matings between *E. coli* and various *Streptomyces* spp. (Bierman *et al.*, 1992; Kieser *et al.*, 2000; Shigeru *et al.*, 2000), including *S. coelicolor* (Paget *et al.*, 1999) and *S. lividans* (van Wezel *et al.*, 2000). This useful vector has not yet been used with *S. scabies.* Finally, pHJ401, a derivative of pHJL400 (S. Jensen, personal communication), is a non-integrating and autonomously replicating plasmid that also has not been tested with *S. scabies.*

The specific objectives of this study were to evaluate different plasmid constructions for efficiency and frequency of introduction into *S. scabies* through PEG-mediated protoplast transformation, electroporation and conjugation. The non-restrictive, non-pathogenic *S. lividans* and the restrictive, non-pathogenic *S. coelicolor* will be included as controls in all experiments. Putative transformants, electrotransformants and transconjugants carrying non-integrative vectors will be verified by plasmid isolation and comparison to the original plasmid. Southern hybridization with probes constructed from the original vector, to blots of digested genomic DNA from putative transformants, electrotransformants, and transconjugants will verify the presence of integrating plasmids.

2.2 Materials and Methods

2.2.1 Bacterial Strains and Growth Conditions

The common scab pathogen, *Streptomyces scabies* strains Lpit10 and Lpit17, were the experimental organisms. The non-pathogenic/permissive *S. lividans* TK64 and the non-pathogenic/restrictive *S. coelicolor* J1501 were included in all experiments as controls (Table 2-1). Plates of sporulating *Streptomyces* were prepared by streaking glycerol spore stocks on YEME solid agar (pH 7.3) (4 g/L Difco yeast extract, 10 g/L malt extract, 4 g/L dextrose, 13 g/L agar) or media specific for each species. The specific medium used for growth of *S. lividans* and *S. coelicolor* was SpMR-Mtl (5 g/L mannitol, 1 g/L Difco yeast extract, 10 g/L $MgCl_2 \cdot 2H_2O$, 4.2 g/L MOPS, 103 g/L sucrose, 22 g/L agar, 20 mM $CaCl_2$, 50 µg/mL histidine, 7.5 µg/mL uracil, 2 mL trace elements solution (pH 7.3)) (S. Jensen, personal communication), while *S. scabies* Lpit 10 and 17 were grown on ISP-4 (Difco, Detroit, MI) medium. Cultures of *Streptomyces* spores for long term storage were prepared by filtering spore suspensions scraped from 1-3 plates through sterile mira cloth (Calbiochem-Novabiochem Corporation, La Jolla, CA), resuspending the spores in 1 mL 20% (w/v) glycerol, and storage at –80°C. All *Streptomyces* liquid cultures were grown in yeast malt extract medium (YEME) (Kieser *et al.*, 2000) or tryptone soy broth (TSB) (Difco) with 1% (w/v) soluble potato starch (Sigma, St. Louis, MO). Liquid cultures were started from one plate of spores washed with 1 mL H_2O into 25 mL of the liquid medium, and grown with vigorous shaking at 30°C. To avoid excessive clumping of *S. lividans* mycelia, a metal spring baffle was included during the liquid culture growth.

Cultures of *Escherichia coli* were grown at 37°C in LB (Luria-Bertani) liquid medium (10 g/L Difco-tryptone, 5 g/L Difco-yeast extract, 5 g/L NaCl), supplemented with the appropriate antibiotic.

2.2.2 Plasmids and Transposons

Plasmids and transposons used in this study are listed in Table 2-2. Antibiotic concentrations used for plasmid maintenance and selection were as follows, unless otherwise noted: 100 µg/mL ampicillin (amp); 50 µg/mL geneticin (gen); 25 µg/mL kanamycin (kan); 25 µg/mL chloramphenicol (cam); 25 µg/mL thiostrepton (tsr);25 µg/mL apramycin (apr) (Sigma). The plasmids were isolated from *E. coli* using the Qiagen mini-prep or midi-prep purification systems (Qiagen, Valencia, CA), depending on the scale of isolation required.

All transposons and plasmids were maintained in *E. coli* DH5a or ED8767. Conjugative plasmids were housed in *E. coli* strain ET12567 (*dam13*::Tn*9 dcm6 hsdM hsdR recF143 zjj201*::Tn*10 galK2 galT22 ara14 lacY1 xyl5 leuB6 thi1 tonA31 rpsL136 hisG4 tsx78 mtli glnV44* F^-), harbouring the driver plasmid pUZ8002 (MacNeil *et al.*, 1992).

The conjugative plasmids were transformed into the *E. coli* strain ET12567/pUZ8002 by electroporation (2.5 KV, 25 µF, 200 O resistance). Immediately after electroporation, 900 µL of SOC medium (20 g/L Difco tryptone, 5 g/L Difco yeast

Table 2-1. List of bacterial species used in this study

Bacterial species	Strain	Source
Streptomyces scabies	Lpit10	H. Lawrence, Agriculture and Agri-Food Canada, Fredericton
S. scabies	Lpit17	H. Lawrence, Agriculture and Agri-Food Canada, Fredericton
S. lividans	TK64	D. Hopwood, John Innes Institute, Norwich, England
S. coelicolor	J1501	S. Jensen, University of Alberta, Edmonton
S. clavuligerus	NRRL 3585	S. Jensen, University of Alberta, Edmonton
Pseudomonas syringae pv. *phaseolicola*	HB9	Agriculture and Agri-Food Canada, Lethbridge
Escherichia coli	ET12567	S. Jensen, University of Alberta, Edmonton
E. coli	DH5a	Gibco-BRL Life Technologies, Bethesda, MD
E. coli	ED8767	N.E. Murray

Table 2-2. Plasmids used in this study

Plasmid	Relevant Characteristics	Host Strain*	Reference/Source
Conjugation			
pSET152	5.5 kb; apr^R lacZa MCS *oriT int*^{FC31} *rep*^{pUC}; integrative	ET12567	Bierman *et al.*, 1992
pKC1218	5.8 kb; apr^R lacZa MCS *oriT rep*^{SCP2*} *rep*^{pUC}; non-integrative, autonomously replicative	ET12567	Bierman *et al.*, 1992
pHJ401	6.5 kb; tsr^R amp^R MCS *oriT*, non-integrative, autonomously replicative	XL1Blue	S. Jensen, University of Alberta, Edmonton
PEG-mediated transformation/ Electroporation			
pJOE2577	9.6 kb; tsr^R amp^R kan^R *tnpA merp* rep; carries Tn*5493*	DH10B	Volff and Altenbuchner, 1997b
Oaac cassette	1.7 kb; gn^R *aacC4*, integrative, housed on pHP45 (4.3 kb)	ED8767	Blondelet-Rouault *et al.*, 1997
pHJL400	5.8 kb; tsr^R amp^R lacZa MCS; non-integrative, autonomously replicative	DH5a	Larson and Herschberger, 1986
pIJ486	6.2 kb; tsr^R MCS; non-integrative, autonomously replicative	*S. lividans* TK64	Ward *et al.*, 1986

*Host species are *E. coli*, unless otherwise stated.

extract, 0.5 g/L NaCl, 2.5 mM KCl, 20 mM glucose, 10mM $MgCl_2$, 10mM $MgSO_4$, pH 7.0) was added. The electro-transformed cultures were transferred to 14 mL snap cap tubes (Becton Dickinson, Franklin Lakes, NJ), grown at 37°C for 1.5 h with shaking (200 rpm), and plated on LA (10 g/L Difco-tryptone, 5 g/L Difco-yeast extract, 5 g/L NaCl, 15 g/mL agar) with selection for the incoming plasmid only, in order to facilitate growth of the newly electro-transformed cultures. Subsequent platings were on LA containing selection for the introduced plasmid, as well as the *dam* mutation (cam) and the pUZ8002 driver plasmid (kan).

2.2.3 Methods for Introduction of Foreign DNA

2.2.3.1 Conjugation

2.2.3.1.1 *Escherichia coli* and *Streptomyces* spp. Preparation

The E. coli ET12567/pUZ8002 cultures carrying the conjugative plasmids (pSET152, pHJ401, pKC1218) were streaked to single colonies on Luria-Bertani solid agar (LA) (10 g/L Difco tryptone, 5 g/L Difco yeast extract, 5 g/L NaCl, 15 g/L agar) with appropriate selection for the transferable plasmid, the dam mutation (cam) and the pUZ8002 driver plasmid (kan). A single colony from these plates was used to inoculate a seed culture in LB containing the same selective antibiotics. After 12-16 h growth, the culture was diluted 1:100 and grown for an additional 4-6 h, until the optical density, as measured on a Beckman DU-65 spectrophotometer (600 nm), reached 0.5-0.6. The cells were washed twice in an equal volume of LB to remove any trace antibiotics that could inhibit initial growth of the transconjugants. Once washed, the cells were gently suspended in 0.1 volume of LB and stored on ice for use in the conjugation.

Separate conjugations were performed with *Streptomyces* spores and mycelia. The procedure in Kieser et al. (2000) specifies one plate of mycelia for each conjugation. However, because of the low transformation efficiency with *S. scabies*, two plates of mycelia were used for each conjugation. *Streptomyces* mycelia started from glycerol spore stocks were grown for 3-4 days to a confluent lawn on MS agar plates (20 g/L mannitol, 20 g/L soya flour (Sigma), 20 g/L agar) (Kieser et al., 2000), without addition of $MgCl_2$. For each conjugation, mycelia were washed from two plates with 2 mL of 20% (v/v) glycerol and collected in 14 mL snap cap tubes (Becton Dickinson). The mycelia were washed by vortexing with 2 mL 20% glycerol, precipitated by centrifugation at 3000 rpm (RT) for 2 min, suspended in 3 mL 20% glycerol with vigorous vortexing, and kept on ice until the *E. coli* cells were prepared.

Preparation of spores for conjugation followed the basic procedure of Kieser et al. (2000) with modifications to optimize the procedure for different strains. Because of the low frequency of transformation with *S. scabies*, approximately 10^9-10^{10} spores, rather than the suggested 10^8 spores, were added to 500 µL 2xYT broth (16 g/L Difco tryptone, 10 g/L Difco yeast extract, 5 g/L NaCl). Spore viabilities at temperatures of 30, 35, 40, 45, and 50°C were tested by incubating aliquots of 1 x 10^9 spores for 10 min at each of the temperatures. Heat-treated spores were plated on YEME agar and incubated at 30°C for 72 h, until individual cultures were visible. Prior to each conjugation the spores were heat-shocked at the most conducive temperature, 40°C, for 10 min and slowly cooled on ice.

2.2.3.1.2 Conjugation Procedure

For each conjugation, 0.5 mL of *E. coli* cells and 0.5 mL of vortexed mycelia or heat-shocked spores were combined in a 1.5 mL microfuge tube. The conjugation was gently mixed by inversion and centrifuged at 3000 rpm and RT for 3 min. Each conjugation was suspended in 100 µL LB and dilutions (10^{-2} and 10^{-3}) were plated on MS agar plates with 10 mM $MgCl_2$ that had been previously dried for 1 h in a laminar flow hood. The putative transconjugants were incubated at 30°C until signs of mycelial growth were visible, generally from 24-48 h. At this time the 25 mL plates were overlaid with 1 mL water containing 0.5 mg nalidixic acid (25 mg/mL) and 1 mg of the appropriate plasmid-selective antibiotic. The plates were further incubated at 30°C for 7-10 days, or until transconjugants were observed. Conjugation experiments were repeated three times with each *Streptomyces* species/plasmid combination.

Transconjugants were picked from the MS plates and plated on species-specific media containing nalidixic acid (25 µg/mL) and appropriate selective antibiotic (25 µg/mL). After sporulation, the transconjugants were streaked twice on selective plates for single colonies and were allowed to sporulate again. Single colonies were finally picked and streaked on selective media to produce a confluent lawn of spores.

2.2.3.2 PEG-Mediated Transformation

2.2.3.2.1 Protoplast Preparation

A heavily sporulating plate of each *Streptomyces* species was used to inoculate 25 mL aliquots of TSB liquid medium. The 250 mL flasks were incubated at 30°C with vigorous shaking (250 rpm) for 30 h. Again, a metal spring was included in the *S. lividans* cultures to prevent large mycelial clumps. Two 25 mL cultures of each species were combined and the mycelia were pelleted by centrifugation at 3000 rpm, RT for 10 min. The combined cultures were suspended in P buffer (Hopwood *et al.*, 1985), supplemented with 1 mg/mL lysozyme (Sigma). The time required for protoplast formation was specific for each species (*S. lividans*, 30 min; *S. scabies*, 45 min; *S. coelicolor*, 60 min). All protoplasting was performed at 25°C with gentle shaking (50 rpm). Protoplast formation was stopped when there were approximately 75% protoplasted and 25% non-protoplasted mycelium in each culture, as determined by phase contrast microscopy (Optiphot-2, Nikon, Tokyo, Japan). The protoplasts were filtered though sterile mira cloth (Calbiochem-Novabiochem Corporation), washed with 5 mL P buffer, pelleted (3000 rpm for 10 min at RT), and decanted. After gentle resuspension (pipetting up and down 3 times) in the residual liquid, the protoplasts were diluted with 5 mL of P buffer and dispensed into 1.5 mL microfuge tubes in 500 µL aliquots. The tubes were placed in a beaker of ice at –80°C to ensure slow freezing.

2.2.3.2.2 Transformation

Immediately prior to transformation, protoplasts were rapidly thawed at 37°C and washed once with 2 mL P buffer. The protoplasts were gently sedimented at 3000 rpm for 6 min and resuspended in the liquid remaining after the supernatant was decanted. One tube containing approximately 10^{10} protoplasts was used for each transformation. All of the following steps took place on ice. For the transformation, 1 µg of plasmid DNA (*Oaac*, pHJL400, pJOE2577 or pIJ486) in 5 µL volume of TE was added to the washed protoplasts, immediately followed by 100 µL of P buffer with 25% (w/v) PEG 1000 (Sigma or NBS Biologicals (Huntingdon, England)). After 1 min, 900 µL of P buffer was added to each reaction. The transformation solution was gently mixed by inversion and pelleted by centrifugation (3000 rpm, 6 min at RT). Each transformation was suspended in 1 mL P buffer and 100 µL of serial dilutions were plated on slightly dry but freshly made (same day) R5GG regeneration plates (103 g/L sucrose, 0.25 g/L K_2SO_4, 10.12

g/L $MgCl_2 \cdot 6H_2O$, 10 g/L glucose, 0.1 g/L casamino acids, 5 g/L yeast extract, 5.73 g/L TES buffer, 200 µL/L R5 trace elements stock (Hopwood *et al.*, 1985), 40 µg/L $FeCl_3 \cdot 6H_2O$, 0.005% (w/v) KH_2PO_4, 20 mM $CaCl_2 \cdot 2H_2O$, 0.3% (w/v) L-proline, 7 mM NaOH).

Control transformations using 5 µL of TE and 5 µL of water were performed along with the test transformation. The TE control was treated as the test transformations, to determine the regeneration frequency of the protoplasts after PEG 1000 exposure. No PEG 1000 was added to the water control in order to determine the regeneration potential of untreated protoplasts. To determine the number of non-protoplasted units 0.01% (w/v) SDS was added to 50 µL of protoplasts (Kieser *et al.*, 2000). Serial dilutions (100 µL) of all controls were plated on slightly dry R5GG regeneration plates. All transformations, including controls, were repeated three times.

The plates were incubated at 30°C for 24-48 h until small colonies were visible, and then overlaid with 3 ml of soft nutrient agar (Hopwood *et al.*, 1985) containing the appropriate selective antibiotic (5 µg/mL tsr, 20 ng/mL gen). The water and TE controls were overlaid with soft agar containing no antibiotic. No overlay was added to the SDS treatments. After the transformants began sporulating, they were transferred to species-specific selective plates. The transformants were purified by three serial single colony transfers, as described for the transconjugants. The pJOE2577 transformants were treated as in Kieser *et al.* (2000), to cure and subsequently verify loss of the temperature-sensitive delivery plasmid.

2.2.3.2.3 Modifications

Various modifications were made to the basic transformation procedure, to improve transformation efficiency. The first modifications involved the conditions used for growth of the mycelial cultures. All of the cultures were grown in YEME and TSB liquid media for 30 or 40 h to maximize the number of highly regenerative protoplasts.

Tests were performed to determine the optimum concentration and duration of lysozyme treatment for protoplast formation. Cultures of the same age (30 h) were subjected to lysozyme concentrations of 0.5 mg/mL, 1 mg/mL, and 1.5 mg/L and incubated for 30, 45, 60, and 90 min. Each combination was plated on slightly dry R5GG regeneration plates, and incubated at 30°C until good growth was observed.

There was the possibility that if the plated dilution of transformed protoplasts were too concentrated, there would be inhibition of regeneration (Engel, 1987; Garcia-

Dominguez *et al.*, 1987). As a result, the transformed mixtures were diluted with P buffer by 10^{-1}, 10^{-2}, or 10^{-3} prior to being plated onto the regeneration media. In an attempt to circumvent the restriction system in *S. scabies* and *S. coelicolor*, the protoplasts were heat-shocked prior to transformation. A series of 10 min heat treatments at 30, 35, 40, 45, and 50°C was conducted with 50 µL aliquots of non-transformed protoplasts of all three species, to determine the temperature for optimum regeneration. The protoplasts were then subjected to this temperature prior to each transformation. The source of PEG 1000 used in transformations can have an influence on transformation efficiency. As a result, two different sources of PEG 1000 were evaluated in the transformations, Sigma and NBS Biologicals.

Traditionally, transformations have been plated on regeneration plates that were dried for 2-4 h to approximately 85% of their original weight (Kieser *et al.*, 2000). The experiments in this study assessed plates that (1) had lost 15% of their original weight, (2) had been dried more extensively (until cracking), and (3) had been left open for approximately 1 h until their surfaces were dry (S. Jensen, personal communication). Transformation efficiency was evaluated for timing of selective soft agar overlay, ranging from an incubation period of 27 h to 48 h after protoplasts were plated.

2.2.3.3 Electroporation

2.2.3.3.1 *Streptomyces* spp. Mycelia Preparation

Mycelia for electroporation were prepared as in Kieser *et al.* (2000), with modifications. To account for the slower growth of the *S. scabies* strains, the CRM (10 g/L glucose, 103 g/L sucrose, 10.12 g/L $MgCl_2 \cdot 6H_2O$, 15 g/L Difco tryptic soy broth, 5 g/L Difco yeast extract) cultures were grown for 30 h at 30°C. During the washing steps, the mycelia were vortexed thoroughly to ensure good separation of the mycelial pellets. After the mycelial pellets were washed, they were resuspended in 4 mL of 30% (w/v) PEG 1000, 10% (w/v) glycerol, 6.5% (w/v) sucrose and divided into 50 µL aliquots. Each mycelial aliquot was rapidly frozen on dry ice/methanol and stored at –80°C.

2.2.3.3.2 Electro-transformation

For each electroporation, 1 µg of plasmid DNA in water was mixed with 50 µL of prepared mycelia thawed on ice. The suspension was transferred to an ice-cold electrocuvette (1 mm gap) (Bio-Rad Laboratories, Richmond, CA), and evenly distributed by gentle tapping of the cuvette. A Gene Pulser (Bio-Rad) was used to provide a 1 kV (10 kV/cm) electric pulse to the solution. The pulse controller was set at a capacity of 25 µF and a resistance of 400 O with a field strength of 8-9 ms. The electroporated mycelia were immediately diluted with 0.75 mL ice-cold CRM, transferred to a 14 mL snap cap tube, and incubated with gentle shaking (100 rpm) for 3 h at 30°C. Transformed cultures were diluted with 200 µL CRM and plated (undiluted, 10^{-1} dilution) on species-specific agar plates containing the appropriate concentration of the required antibiotic. As with the other procedures, each transformation was repeated three times. Putative transformants were grown at 30°C and serially transferred three times onto selective media in order to isolate single sporulating colonies.

2.2.3.3.3 Modifications

Modifications of the basic protocol for electroporation were made. The DNA concentration for each electroporation was varied from 1 µg to 10 µg in a 5 µL volume of water. The second modification was the choice of regeneration media for the electroporated mycelia. In addition to the suggested TSB agar plates (30 g/L Difco tryptic soy broth, 15 g/L agar), species-specific media (see section 2.1) were also used to plate the serial dilutions from each transformation.

2.2.4 DNA Manipulations

2.2.4.1 DNA Isolation

Sporulating plates of individual transformants, electro-transformants, and transconjugants were washed with water and used to inoculate 10 mL TSB + 1% (w/v) potato starch (Sigma) containing the appropriate selective antibiotic (Table 2-2). Nalidixic acid at concentrations of 25 or 50 µg/mL was added to liquid cultures of the transconjugants. All cultures were grown at 30°C with vigorous shaking (250 rpm) until there was extensive mycelial growth, usually for 72-98 h. Cultures were generally removed from the incubator before they had produced an excessive amount of pigment, to facilitate DNA isolation. In order to remove any potentially inhibitory factors, the mycelia were washed with 2 mL TEGLR (25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 50 mM glucose), without lysozyme. The mycelia were suspended in 1.4 mL TEGLR and frozen at –20°C. To facilitate lysis, the cultures were freeze-thawed (-20°C/60°C) for six cycles.

Non-integrating plasmids were isolated from the recipient *Streptomyces* spp. following the alkaline lysis and potassium acetate precipitation procedure in Kieser *et al.* (2000). The phenol (pH 8) extraction was performed, however the optional second acid phenol extraction and its accompanying steps were omitted. Following the first isopropanol precipitation, the DNA was dissolved in 1 mL TE, 0.3 M sodium acetate (pH 5.2) and 10 µg t-RNA. Two volumes of 95% (v/v) ethanol were added and the mixture was incubated at room temperature for 1 h. Finally, the DNA was pelleted by centrifugation at 12 000 rpm for 10 min (RT), washed with 70% (v/v) ethanol, and dissolved in 200 µL TE.

Genomic DNA, and the integrated plasmids, was isolated following the rapid small-scale procedure in Hopwood *et al.* (1985), with modifications. As with the plasmid isolation, the mycelia were suspended in TEGLR and freeze-thawed repeatedly. Lysis was promoted by mixing the solution well after addition of 2% (w/v) SDS using 5 mL large bore transfer pipettes (Fisher Scientific, Nepean, ON). To limit the possibility of shearing the DNA, the solution was not vortexed during the phenol-chloroform extraction; rather it was gently mixed with a transfer pipette. The two precipitation steps were performed without addition of spermine-HCl. The DNA pellets were each dissolved in 200 µL TE.

2.2.4.2 DNA Digests and Blots

2.2.4.2.1 Restriction Endonuclease Digestion

Non-integrating plasmid DNA (pHJL400, pHJ401, pKC1218, and pIJ486) isolated from putative transformants, electrotransformants, and transconjugants was digested with restriction endonucleases (*Xho*I and *Bam*HI) following the manufacturer's instructions (Gibco-BRL, Burlington, ON). The linearized plasmids were resolved on a 0.7% (w/v) ultrapure agarose gel (Gibco-BRL), to verify that the isolated plasmids were the same size as the original transforming plasmid. The plasmid pIJ486 (6.2 kb) isolated from *S. lividans* was included as a control to verify the reliability of plasmid isolation.

Streptomyces genomic DNA isolated from putative pSET152 transconjugants, as well as pJOE2577 and *Oaac* transformants/electrotransformants was digested with *Bgl*II (New England Biolabs, Beverly, MA). Non-transformed *Bgl*II-digested *S. lividans*, *S. coelicolor* and *S. scabies* genomic DNA were included as negative controls in all analyses. Bovine serum albumin (BSA) (100 µg/mL) and spermidine (2 mM) were included to facilitate digestion. Ten units of enzyme were added and the reaction was incubated at 37°C for 1 h, an additional ten units of enzyme were added and the reaction was incubated overnight. The DNA fragments were separated on a 0.7% (w/v) agarose gel run at 15 V for 16 h.

2.2.4.2.2 Southern Hybridization

Digested DNA was depurinated in 0.4 M HCl and transferred overnight by capillary action in 0.4 M NaOH onto Hybond N+ membranes (Amersham Pharmacia, Buckinghamshire, England). The DNA was fixed to the membranes by a UV Stratalinker following the manufacturer's instructions (Stratagene, LaJolla, CA). The membranes were pre-hybridized in pre/post hybridization buffer (1% (w/v) sarkosyl, 200 mM NaCl, 10 mM Tris-HCl (pH 7.5-8.0), 1 mM EDTA) for 45 min at 65°C, and then in the hybridization solution (1% sarkosyl, 1 M NaCl, 50 mM Tris (pH 7.5), 10% (w/v) dextran sulfate added at time of use) for 30 min at 65°C. The pSET152, *Oaac* and pJOE2577 vectors were randomly labelled using the Canadian Life Tech Kit (Canadian Life Technologies, Burlington, ON), and the probes were added to the hybridization solutions at 1×10^6 cpm/µL and the blots hybridized for 16-18 h at 65°C. Blots were washed for 5 min at 65°C in pre/post buffer, followed by two 20 min washes in 1X SSC and a final 20 min wash in 0.5X SSC, all at 65°C. Autoradiographs were prepared by exposing the membrane to Fuji SuperRX film (Fuji, Tokyo Japan) using a Biomax intensifying screen (Eastman Kodak, Rochester, NY) at –80°C for 4 h.

2.3 Results

2.3.1 Growth Conditions

Traditionally, liquid *Streptomyces* cultures are grown in YEME media and spores are propagated on YEME plates. However, the conditions that facilitate the most successful growth vary from strain to strain. As a result, various types of media were investigated in order to optimize growth of the cultures used in this study. To rapidly identify the conditions most conducive to growth, *Streptomyces* spp. cultures were grown in the different media (liquid and solid) during the preliminary experiments. The medium that resulted in an obvious difference in growth was identified and utilized for the remainder of the experiments. Liquid TSB resulted in the most rapid growth, and generated the greatest mycelial mass. Cultures raised on species-specific solid agar plates (Spm-RTL for *S. coelicolor* and *S. lividans*, and ISP-4 for *S. scabies*) allowed for the heaviest sporulation.

Overall the *Streptomyces* spp. cultures grown for 40 h generated the greatest number of protoplasts (Table 2-3). The difference between 30 h and 40 h S. *scabies* Lpit17 cultures, for most incubation times (30, 45 and 75 min), was significant (p=0.05) as determined by the Tukey's w pairwise comparisons. However, the length of incubation was generally not significant for the other species. All subsequent data presented in this study were analysed for significant differences with Tukey's test.

2.3.2 Lysozyme Treatment

The conditions for lysozyme treatment of the mycelia were also optimized. The greatest number of protoplasts was obtained, for all species, when 40 h cultures were digested for 75 min (Table 2-3). The number of protoplasts formed was only significantly different (p=0.05) for the *S. scabies* spp. Even though a 75 min lysozyme digestion of the 40 h cultures led to the highest number of protoplasts, the 30 h cultures allowed for the best regeneration of protoplasts. The difference in regeneration between the 30 h and 40 h cultures was easily determined visually. As a result, 30 h cultures were used for protoplast formation. The results in Table 2-4 show the highest regeneration obtained for each species. Each culture was 30 h old and digested with 1mg/mL of lysozyme. The optimal duration of lysozyme digestion varied for each species; 30 min for *S. lividans*, 45 min for *S. scabies*, and 60 min for *S. coelicolor*. Regeneration of *S. lividans* was significantly greater (p=0.05) than regeneration of the other three *Streptomyces* strains. These conditions were used for all transformation experiments.

2.3.3 Antibiotic Sensitivity

In order to determine the optimum concentration of antibiotic in the soft agar overlay used for transformations, antibiotic sensitivity assays were performed. Plates were rated as growth or no-growth, and the lowest antibiotic concentration to inhibit colony growth was utilized in all subsequent experiments. All species were resistant up to 300 µg/mL amp. Concentrations of 3 µg/mL of tsr were inhibitory to all strains. Geneticin inhibited growth of all three species at a concentration of 20 µg/mL. Apramycin was more effective at limiting *S. scabies* growth (5µg/mL) than *S. lividans* and *S. coelicolor* (25 µg/mL).

Table 2-3. Protoplast formation after lysozyme treatment of 30 and 40 h *Streptomyces* spp. cultures

***Streptomyces* spp.**		**Culture Age (h)**		**Lysozyme (min)**		**Protoplasts[*] (number/mL)**
S. lividans	A[**]	30	R[+]	30	W[++]	8.3 x 10^9
	A		R	45	WX	8.6 x 10^9
	A		R	60	XY	9.3 x 10^9
	A		R	75	Y	9.8 x 10^9
	A	40	R	30	W	9.0 x 10^9
	A		S	45	W	9.4 x 10^9
	A		R	60	W	9.5 x 10^9
	A		R	75	W	1.0 x 10^{10}
S. coelicolor	AB	30	R	30	W	7.5 x 10^9
	A		R	45	WX	7.9 x 10^9
	B		R	60	WX	8.2 x 10^9
	B		R	75	X	8.7 x 10^9
	B	40	R	30	W	7.9 x 10^9
	B		R	45	W	8.2 x 10^9
	B		R	60	W	8.7 x 10^9
	B		R	75	W	8.8 x 10^9
S. scabies Lpit10	B	30	R	30	W	7.2 x 10^9
	B		R	45	X	9.0 x 10^9
	A		R	60	Y	9.8 x 10^9
	A		R	75	Z	9.8 x 10^9
	C	40	R	30	W	7.4 x 10^9
	A		S	45	W	9.6 x 10^9
	AB		R	60	X	9.8 x 10^9
	C		R	75	X	1.6 x 10^{10}
S. scabies Lpit17	B	30	R	30	W	6.3x 10^9
	C		R	45	W	6.9 x 10^9
	B		R	60	X	7.9 x 10^9
	B		R	75	X	8.5x 10^9
	C	40	S	30	W	7.6 x 10^9
	B		S	45	WX	8.3 x 10^9
	C		R	60	XY	8.6 x 10^9
	D		S	75	Y	9.3 x 10^9

(Continued on page 52)

(Continued from page 51)

[*] Protoplast numbers were averaged over three independent experiments and were calculated from average counts in five 1/400 mm^2 haemocytometer squares. They are expressed as the number of protoplasts per mL.
Different letters indicate a significant difference ($p=0.05$) in the number of protoplasts determined by Tukey's test.

[**] A-D Comparison of protoplast number at different culture ages and lysozyme incubation times between each species.

[+] R-S Comparison of protoplast number after different lysozyme incubation times for 30 and 40 h cultures within each species.

[++] W-Y Comparison of protoplast number after different lysozyme incubation times within each culture age.

Table 2-4. Regeneration efficiencies of 30 h *Streptomyces* spp. cultures

***Streptomyces* spp.**		**Regeneration Efficiency** *
Streptomyces lividans	A **	1.9×10^{-1}
S. coelicolor	B	4.1×10^{-2}
S. scabies Lpit10	B	3.5×10^{-2}
S. scabies Lpit17	B	3.0×10^{-2}

* Efficiencies were based on three independent experiments and were calculated by expressing the number of regenerated protoplasts obtained over the number of protoplasts plated.

** A-B Different letters indicate a significant difference ($p=0.05$) in regeneration efficiency between each species determined by Tukey's test.

2.3.4 Heat Treatment

In order to initiate germination and enhance conjugative DNA transfer, spores of all three *Streptomyces* spp. were incubated at a range of temperatures (30°C-50°C) and their viability after exposure to each temperature was determined (Table 2-5). To circumvent the restriction systems of both *S. scabies* and *S. coelicolor*, protoplasts were tested for their ability to survive exposure to the same range of temperatures (Table 2-6). In all cases, *S. lividans* spores showed significantly (p=0.05) higher survivability. The spores from all three *Streptomyces* spp. had good survival at temperatures from 30°C-45°C, however, there was a significant (p=0.05) decrease after 40°C. At the recommended incubation temperature of 50°C (Engel, 1987), the viability of the spores was significantly (p=0.05) lower than the other temperatures.

Protoplast regeneration of all species was similar to spore viability, with protoplasts of *S. lividans* consistently showing significantly (p=0.05) greater regeneration (Table 2-6). For all three bacterial species, significantly (p=0.05) less regeneration was seen between those protoplasts incubated at 30°C and those incubated at the other temperatures. However, good protoplast regeneration was obtained for all species up to 40°C. No protoplasts survived exposure to temperatures of 45°C and 50°C. Based on the results of these experiments, 40°C was chosen as the optimum temperature for *S. lividans*, *S. coelicolor*, and *S. scabies* protoplast and spore incubation.

2.3.5 DNA Introduction

2.3.5.1 Conjugation

Exconjugants were obtained for all three *Streptomyces* species, but frequencies were significantly (p=0.05) different depending on the plasmid that was introduced. Significantly (p=0.05) more pSET152 exconjugants were generated for each species tested.

2.3.5.1.1 Effect of Conjugative Material

Initial conjugations were performed using either *Streptomyces* spores or mycelia; in all cases significantly (p=0.05) more exconjugants were obtained when mycelia was used. Table 2-7 shows the number of exconjugants obtained when plasmid pSET152 was introduced into prepared mycelia or spores of all three *Streptomyces* species. The other

Table 2-5. Effect of heat treatment on the viability of *Streptomyces* spp. spores

***Streptomyces* spp.**		**Temperature (°C)**		**Percent Viability * (%)**
S. lividans	A [**]	30	R [+]	73
	A	35	RS	70
	A	40	ST	60
	A	45	TU	58
	A	50	V	28
S. coelicolor	B	30	R	60
	B	35	R	59
	B	40	S	46
	B	45	S	45
	B	50	T	17
S. scabies Lpit10	BC	30	R	57
	BC	35	RS	51
	BC	40	S	44
	B	45	T	13
	C	50	U	2×10^{-7}
S. scabies Lpit17	C	30	R	52
	C	35	R	49
	C	40	S	36
	C	45	T	24
	C	50	U	1.0×10^{-7}

[*] Mean values, based on three independent experiments, are expressed as the number of colonies obtained after heat treatment over the number of colonies obtained with no heat treatment.

Different letters indicate a significant difference (p=0.05) in percent viability determined by Tukey's test.

[**] A-C Comparison of percent viability at different incubation temperatures between each species.

[+] R-V Comparison of percent viability at different incubation temperatures within each species.

Table 2-6. Regeneration efficiencies of *Streptomyces* spp. protoplasts after heat treatment

***Streptomyces* spp.**		**Temperature (°C)**		**Regeneration Efficiency**[*]
Streptomyces lividans	A[**]	30	R[+]	3.4×10^{-1}
	A	35	S	7.8×10^{-3}
	A	40	S	6.3×10^{-3}
		45	S	0
		50	S	0
S. coelicolor	B	30	R	5.4×10^{-2}
	B	35	S	1.9×10^{-2}
	A	40	T	5.7×10^{-3}
		45	T	0
		50	T	0
S. scabies Lpit10	B	30	R	4.5×10^{-2}
	A	35	S	6.7×10^{-3}
	B	40	ST	3.5×10^{-3}
		45	ST	0
		50	T	0
S. scabies Lpit17	B	30	R	4.1×10^{-2}
	AB	35	S	1.1×10^{-2}
	B	40	T	2.6×10^{-3}
		45	T	0
		50	T	0

[*] Efficiencies are based on three sets of incubations at each temperature, and are calculated as the number of regenerated protoplasts obtained over the number of total protoplasts used.

Different letters indicate a significant difference ($p=0.05$) in regeneration efficiency determined by Tukey's test.

[**] A-B Comparison of regeneration efficiency at different incubation temperatures between each species.

[+] R-T Comparison of regeneration efficiency at different incubation temperatures within each species.

Table 2-7. Number of *Streptomyces* spp. exconjugants obtained when pSET152 was conjugally transferred into spores or mycelia

Streptomyces spp.	Number of Exconjugants[*] Mycelia		Spores	
Streptomyces lividans	1.0×10^5	A[**]	2.9×10^3	B
S. coelicolor	8.4×10^4	A	6.3×10^3	B
S. scabies Lpit10	1.8×10^4	A	1.0×10^3	B
S. scabies Lpit17	2.4×10^4	A	1.0×10^3	B

[*] Numbers of exconjugants were determined by averaging three independent conjugation experiments, each utilizing plasmid pSET152 and either mycelia or spores as the conjugative material.

Different letters indicate a significant difference (p=0.05) in number of exconjugants determined by Tukey's test.

[**] A-B Comparison of number of exconjugants obtained using mycelia and spores for each species.

conjugative vectors, also generated more exconjugants when mycelia was used as compared to spores (data not shown). Although spores of *S. lividans* were significantly (p=0.05) easier to conjugate than spores of the other species, there was no difference between the mycelia of *S. lividans* and *S. coelicolor*. For both mycelia and spores, the number of *S. scabies* Lpit10 and Lpit17 exconjugants were significantly (p=0.05) less than the number obtained with *S. lividans* and *S. coelicolor*. Based on these results, mycelia was used as the conjugative material for the remainder of the experiments. Increasing the amount of mycelia used in each conjugation (four plates of mycelia rather than two) did not result in a significant increase in the number of exconjugants, so the smaller mycelial mass was used throughout the experiments (data not shown).

2.3.5.1.2 Effect of the Vectors

Of the three conjugative vectors used (Table 2-2) only pSET152 was successfully introduced into *S. scabies*. Plasmid pSET152 exconjugants of both *S. lividans* and *S. coelicolor* were generated in a significantly (p=0.05) greater number than those of *S. scabies* (Table 2-8). The results of conjugation with pSET152 are shown in Figure 2-1. The integrative vector is detected in each of the exconjugants from all three species, including the two strains of *S. scabies*. Control cultures (not conjugated), lanes 13-16, lack a hybridization signal to pSET152. The similarity of hybridization pattern among the individual exconjugants within each species indicates that this plasmid integrated into a specific site, as expected.

The two non-integrating conjugative vectors, pKC1218 and pHJ401, were successfully introduced into *S. lividans* and *S. coelicolor* (Figure 2-2), but not into *S. scabies*. There was no significant difference between the number of *S. lividans* and *S. coelicolor* exconjugants obtained with pKC1218. Plasmid pHJ401 resulted in significantly (p=0.05) more *S. lividans* than *S. coelicolor* exconjugants (Table 2-8). The plasmids isolated from the exconjugants, lanes 7-10 (pHJ401) and lanes 17-20 (pKC1218), were comparable in size when compared to the original plasmids in lanes 6 and 16 respectively. For both pHJ401 and pKC1218 this is indicative of bona fide exconjugants.

2.3.5.2 PEG-Mediated Transformation

Transformations with the non-integrative pHJL400 and pIJ486 vectors, and the integrative pJOE2577 and *Oaac* transposons were successful for *S. lividans* and

Table 2-8. Number of *Streptomyces* spp. exconjugants generated from introduction of the conjugative vectors

***Streptomyces* spp.**		**Plasmid**		**Number of Exconjugants**[*]
Streptomyces lividans	A[**]	pSET152	R[+]	1.3×10^5
	A	pKC1218	S	7.6×10^4
	A	pHJ401	T	1.2×10^5
S. coelicolor	AB	pSET152	R	8.0×10^4
	A	pKC1218	R	8.0×10^4
	B	pHJ401	S	7.3×10^4
S. scabies Lpit10	B	pSET152	R	1.8×10^4
	B	pKC1218	S	0
	C	pHJ401	S	0
S. scabies Lpit17	B	pSET152	R	2.0×10^4
	B	pKC1218	S	0
	C	pHJ401	S	0

[*] Mean number of exconjugants from three independent conjugation experiments. Different letters indicate a significant difference ($p=0.05$) determined by Tukey's test.

[**] A-C Comparison of number of exconjugants obtained using different plasmids between each species.

[+] R-T Comparison of number of exconjugants obtained using different plasmids within each species.

Figure 2-1. Agarose gel (top) and autoradiogram (bottom) of exconjugants containing the site-specific integrative vector pSET152. Genomic DNA was isolated from each putative exconjugant, digested with *Bgl*II, separated by size on a 0.7% (w/v) agarose gel and the gel blotted to nylon membrane. The blot was probed with ^{32}P-labelled pSET152. Lanes 1-3 contain exconjugants of *Streptomyces lividans*, 4-6 contain exconjugants of *S. coelicolor*, 7-9 contain exconjugants of *S. scabies* Lpit10, and 10-12 contain exconjugants of *S. scabies* Lpit17. Lanes 13-16 are non-transformed controls of *S. lividans*, *S. coelicolor*, *S. scabies* Lpit10, and *S. scabies* Lpit17, respectively. Lane M is ? *Hin*dIII marker. Size in Kb is indicated on the side.

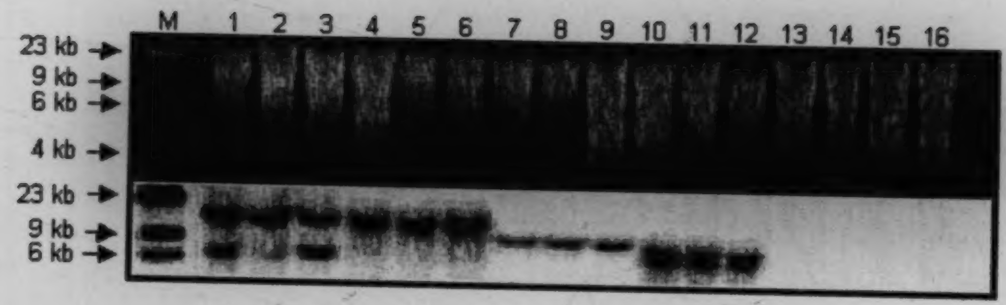
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Figure 2-2. Agarose gel of non-integrating plasmids isolated from the exconjugants, transformants and electrotransformants. The introduced plasmids were isolated, linearized with an appropriate restriction enzyme, and run on a 0.7% (w/v) agarose gel. The lanes are as follows: pHJL400 (lanes 1-5) with the pHJL400 original plasmid control (lane 1); lanes 2 and 4 pHJL400 electrotransformants of *Streptomyces lividans* and *S. coelicolor*, respectively; lanes 3 and 5 pHJL400 transformants of *S. lividans*, and *S. coelicolor*, respectively; pHJ401 (lanes 6-10) with lane 6 the original pHJ401 plasmid (control); lanes 7 and 8 pHJ401 exconjugants of *S. lividans*; and lanes 9 and 10 pHJ401 exconjugants of *S. coelicolor*; pIJ486 (lanes 11-15) with lane 11 the original pIJ486 plasmid (control); lanes 12 and 14 pIJ486 electrotransformants of *S. lividans* and *S. coelicolor*, respectively; lanes 13 and 15 pIJ486 transformants of *S. lividans*, and *S. coelicolor*, respectively; pKC1218 (lanes 16-20) with lane 16 the original pKC1218 plasmid (control); lanes 17 and 18 pKC1218 exconjugants of *S. lividans*; and lanes 19 and 20 pKC1218 exconjugants of *S. coelicolor*. DNA ladder (1 kb) is loaded in lane M. Size in Kb is indicated on the side.

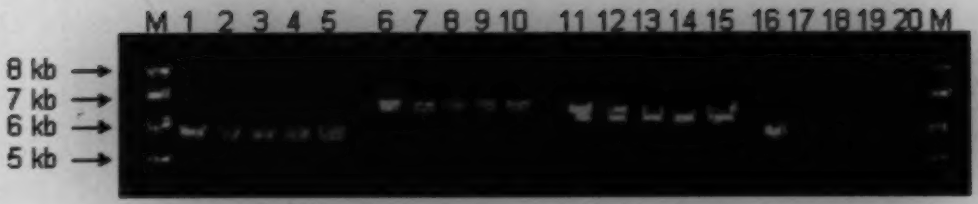
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Table 2-9. Efficiency and frequency of transformation of *Streptomyces* spp. with different plasmids

***Streptomyces* spp.**		**Plasmid**		**Transformation Efficiency** [*]	**Transformation Frequency** [+]
Streptomyces lividans	A [**]	pJOE2577	R [++]	1.0×10^{-6}	1.0×10^{4}
	A	*Oaac*	S	1.0×10^{-5}	1.0×10^{5}
	A	pIJ486	T	2.1×10^{-5}	2.1×10^{5}
	A	pJHL400	RS	8.1×10^{-4}	8.1×10^{4}
S. coelicolor	B	pJOE2577	R	5.0×10^{-8}	0.5×10^{3}
	A	*Oaac*	S	6.1×10^{-6}	6.1×10^{4}
	B	pIJ486	T	4.2×10^{-4}	4.2×10^{4}
	B	pJHL400	U	6.1×10^{-6}	6.1×10^{4}

All values are an average of three separate transformation experiments.

[*] Efficiencies were calculated by expressing the number of transformants obtained over the number of total protoplasts used in each reaction.

[+] Frequencies were expressed as the number of transformants/μg DNA.

Different letters indicate a significant difference (p=0.05) determined by Tukey's test.

[**] A-B Comparison of transformation efficiency/frequency with different plasmids between each species.

[++] R-U Comparison of transformation efficiency/frequency with different plasmids within each species.

S. coelicolor (Table 2-9), but not for *S. scabies*. All transformations contained <0.1% non-protoplasted units, as determined by plating aliquots of the protoplasts treated with SDS.

2.3.5.2.1 Effect of Modifications

The total number of colonies obtained when 10^{-1}, 10^{-2} or 10^{-3} dilutions of transformed protoplasts were plated for regeneration was proportional to the serial dilutions. Inhibition of protoplast regeneration at the lower dilutions did not occur; therefore this dilution series was plated in subsequent experiments and the frequencies of transformation were calculated using these values.

The putative transformants were initially plated on regeneration plates that were dried (1) to cracking, (2) until 15% of their original weight was lost, or (3) until the plate surface was dry. There was no difference in the total number of transformants obtained with the three different types of plates. Transformation results are presented in Table 2-9. The remaining transformations were plated on surface-dried regeneration media, to expedite the screening process.

Transformation plates were overlaid with selective soft agar between 27 and 48 h. The timing was dependent on appearance of the first colonies on the regeneration plates, although plates were generally ready for overlay at 48 h. The time of overlay (27 or 48 h) did not influence the number of putative transformants obtained, so long as the colonies were visible (Table 2-9). Plates that were overlaid when the colonies were not yet visible to the naked eye, tended to yield no potential transformants.

2.3.5.2.2 Effect of the Vectors

The vectors (Table 2-2) were all successfully introduced into *S. lividans* and *S. coelicolor*. Figure 2-2 shows the non-integrating plasmids pHJL400 and pIJ486 used in transformation. As with the exconjugants, transformants containing plasmids of the same size as the original introduced plasmid verifies an actual transformant.

The efficiency of transformation was calculated by dividing the number of transformants obtained in the experiment by the total number of protoplasts used for each transformation, and the frequency calculated by dividing the number of transformants over the amount of DNA (1 µg) used for each transformation. The transformation efficiencies and frequencies for *S. lividans* and *S. coelicolor* with the different plasmids are indicated in Table 2-9. Transformation efficiency and frequency of plasmids pJOE2577 (isolated from *E. coli* DH10B), pIJ486 (isolated from *S. lividans* TK64) and pHJL400 (isolated from *E. coli* DH5a) were significantly (p=0.05) higher for *S. lividans* than for *S. coelicolor*. The *Oaac* integrative transposon (isolated from *E. coli* ED8767) was transformed into *S. lividans* and *S. coelicolor* with equal efficiency. The integrative transposon pJOE2577 had a significantly (p=0.05) lower level of transformation efficiency to both *S. lividans* and *S. coelicolor* than the other plasmids.

Transposition of *Oaac* was verified by Southern hybridization, using the transposon cassette as a probe (Figure 2-3). All of the selected putative transformants showed hybridization to the transposon. Transformation of Tn*5493* from pJOE2577 was detected by replica plating putative transformants confirm tsr resistance arising from integration of Tn*5493* into the chromosome. The transformation frequencies are shown in Table 2-9. The transformed colonies were then replica plated with kan selection to test for loss of the delivery plasmid. The colonies were all kan^s, indicating loss of the delivery plasmid and transposition of Tn*5493*. Transposition of Tn*5493* into the chromosome was then verified by Southern hybridization of digested genomic DNA that were hybridized with radioactively labelled probes of the Tn*5493* transposon alone, and the complete vector (Figure 2-4). No hybridization occurred between the untransformed controls and either the *Oaac* or Tn*5493* probes (data not shown).

Based on the hybridization patterns from Figure 2-4 there is some site specificity for transposon integration, rather than a completely random insertion. Non-random insertion of Tn*5493* is evident in lanes 4, 5, 7, and 10.

2.3.5.3 Electroporation

Electroporation generated true electrotransformants for *S. lividans* and *S. coelicolor* with specific plasmids (Table 2-10). Electrotransformation was not successful at introducing any plasmid/vector into *S. scabies*.

2.3.5.3.1 Effect of Regeneration Media

Putative transformants were plated on both the traditional MS agar with $MgCl_2$ and species-specific media. There was no improvement in electrotransformation efficiency between the two different medias (data not shown).

2.3.5.3.2 Effect of Vectors

The frequency of electroporation was significantly ($p=0.05$) lower than that for traditional PEG-mediated transformation, for all plasmids introduced (compare Table 2-9 and Table 2-10). The non-integrative pHJL400 and pIJ486 vectors and the integrative *Oaac* cassette were successfully introduced into *S. lividans* and *S. coelicolor*, although the occurrence of electrotransformants was generally low ($0.5\text{x}10^1$ – $2\text{x}10^1$ colonies/µg DNA). The electrotransformation frequencies of *S. lividans* by plasmids pJHL400 and pIJ486, were significantly ($p=0.05$) greater than when *S. coelicolor* was the recipient. This same trend was seen with the transformations. Neither species was electrotransformed with integrative pJOE2577.

The autonomously replicating plasmids pHJL400 and pIJ486 and the integrating plasmid *Oaac*, isolated from electroporated transformants can be seen in Figure 2-2 and 2-3, respectively. As with the traditional transformations, the electrotransformants contained plasmids that were the same size as the original plasmids, indicating successful electrotransformation. Transposition of the integrative *Oaac* cassette after electroporation was verified in the same manner as the transformants.

2.4 Discussion

In order to identify, characterize and verify gene function in *S. scabies*, it is imperative to find vectors and methods for DNA introduction. The original intent of this project was to use transposon mutagenesis to introduce simple site mutations and identify pathogenicity genes within *S. scabies*. Gene inactivation caused by exogenous DNA insertion avoids the multiple mutations caused by chemical or UV methods (Anderson, 1995). Thus, the phenotypic change caused by this single site mutation can be more readily identified.

When it became apparent that transposon vectors could not be introduced into *S. scabies* by PEG-mediated transformation, alternative methods were explored for introduction of foreign DNA into *S. scabies*. Traditional PEG-mediated transformation and electroporation were unsuccessful for introduction of any of the selected vectors into *S. scabies*. Alternatively, conjugation facilitated successful introduction of foreign DNA into *S. scabies*, in a vector-dependent manner. This is the first report of DNA introduction, through any method, into *S. scabies*.

Cultures of *S. lividans* and *S. coelicolor* were included as controls in all experiments. The positive control *S. lividans* is a non-restricting, non-pathogenic strain

Figure 2-3. Agarose gel (top) and autoradiogram (bottom) of putative transformants and electrotransformants containing the *Oaac* transposon. Genomic DNA was isolated, digested with *Bgl*II, size-fractionated on a 0.7% (w/v) agarose gel, and blotted to nylon membrane. The genomic blot was probed with a ^{32}P-labelled *Oaac* cassette. The lanes are as follows *Streptomyces lividans* transformants (lane 1 and 2), *S. lividans* electrotransformant (lane 3), *S. coelicolor* transformant (lane 4), *S. coelicolor* electrotransformants (lane 5). Lane M is *Hin*dIII-digested ? DNA. Size in Kb is indicated on the side.

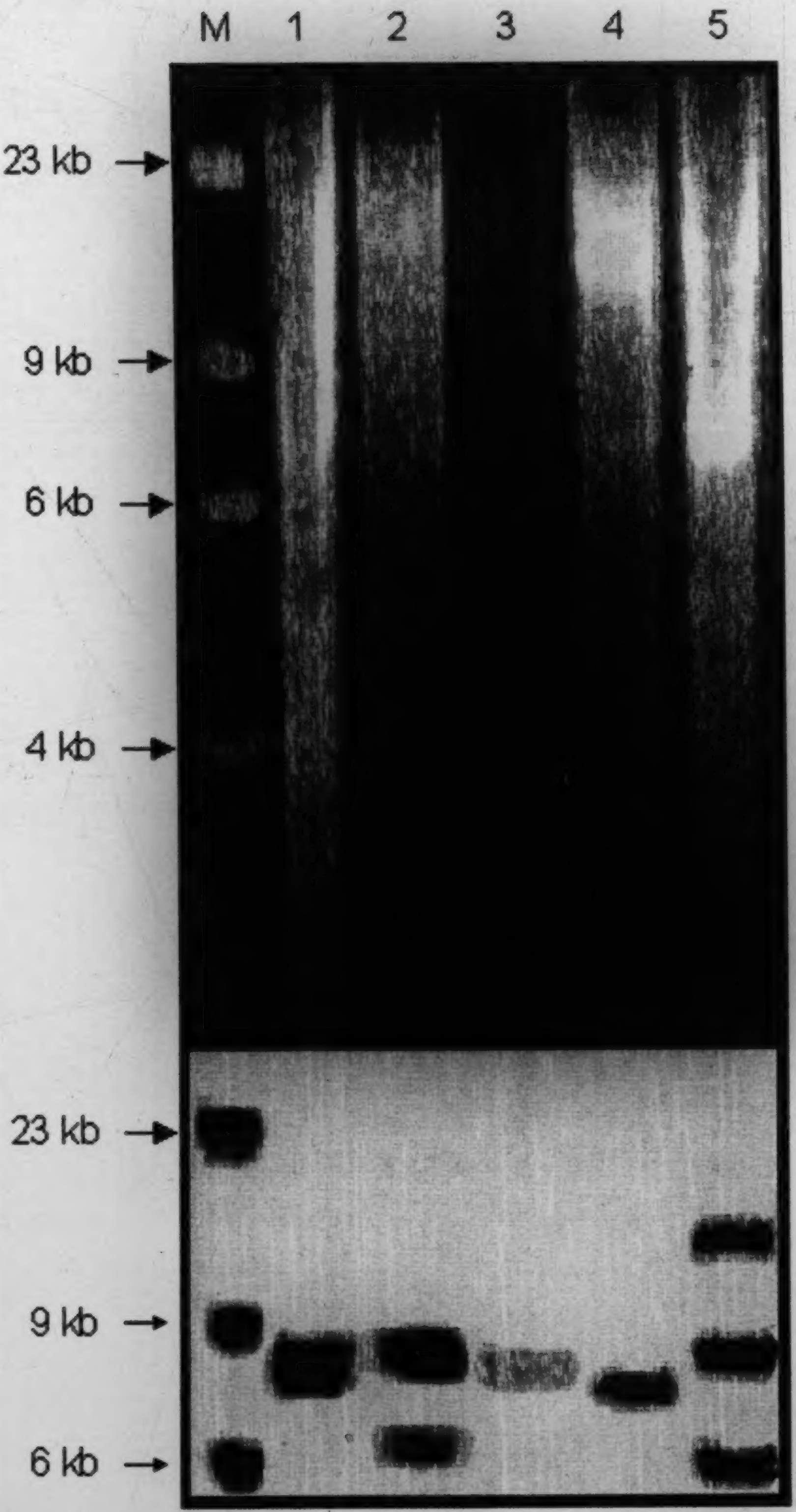
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Figure 2-4. A 0.7% (w/v) agarose gel (top) was used to separate *Bgl*II-digested genomic DNA of putative *Streptomyces lividans* and *S. coelicolor* pJOE2577 transformants. Digested genomic DNA was hybridized with ^{32}P-labelled Tn*5493*; the corresponding autoradiogram is aligned below the agarose gel. The lanes are as follows *S. lividans* transformants (lanes 1-6) and *S. coelicolor* transformants (lanes 7-12). Lane M is *Hin*dIII-digested ? DNA. Size in Kb are indicated on the side.

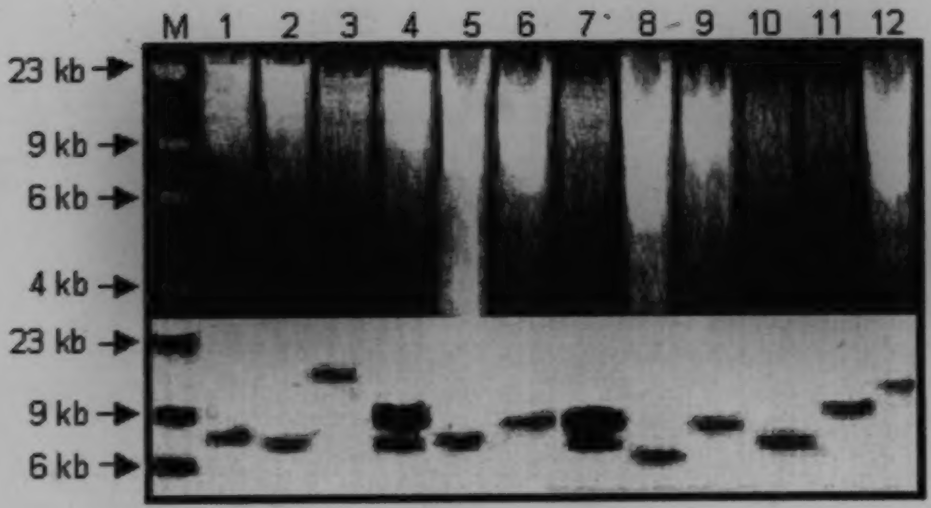
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Table 2-10. Electroporation efficiency of *Streptomyces* spp. using different plasmids

***Streptomyces* spp.**		**Plasmid**		**Electroporation Frequency**[*]
Streptomyces lividans	A[**]	pJOE2577	R[+]	0
	A	*Oaac*	R	2.0×10^1
	A	pIJ486	R	1.2×10^1
	A	pJHL400	S	1.1×10^1
S. coelicolor	A	pJOE2577	R	0
	A	*Oaac*	R	1.0×10^1
	B	pIJ486	RS	0.5×10^1
	B	pJHL400	S	1.0×10^1

[*] Values are the averages of three independent experiments, reported as the number of colonies/μg DNA.

Different letters indicate a significant difference determined by Tukey's test.

[**] A-B Comparison of electroporation frequency with different plasmids between each species.

[+] R-S Comparison of electroporation frequency with different plasmids within each species.

that is known to be easily transformable and is widely used in genetic analysis. The known restrictive species, *S. coelicolor* was included as a negative control for DNA introduction. The presence of the restriction system in *S. coelicolor* makes standard methods of DNA introduction difficult. As a result, any protocol successful at introducing DNA into *S. coelicolor* would indicate conditions that are able to promote DNA introduction under the constraints imposed by its specific restriction system.

Even though there was some vector specificity, all of the methods of DNA introduction used in this study led to transformed colonies of both *S. lividans* and *S. coelicolor*. All of the integrative (pJOE2577, *Oaac*, and pSET152) and non-integrative (pHJL400, pHJ401, pKC1218, and pHJ486) plasmids were introduced into both of the control species. The results reported here verify these plasmids are useful in genetic analysis of *Streptomyces* spp.

2.4.1 Transformation

Transformation frequencies of *S. lividans* and *S. coelicolor* (Table 2-9), during the course of these experiments, were comparable to that previously reported (Bibb *et al.*, 1978; Oh and Chater, 1997; Volff and Altenbuchner, 1997b). There have been no reported studies of *S. scabies* transformation. The significantly ($p=0.05$) higher transformation frequency of *S. lividans* than that of *S. coelicolor* was anticipated, due to the lack of a restriction system in this species. Transformations of *S. coelicolor* with various plasmids were successful, but with efficiencies adversely affected by the restriction/modification system. Successful transformation of *S. coelicolor* was most likely facilitated by the design of the plasmids, which were such that the restriction system(s) did not recognize enough potential restriction sites to eliminate all transformation events. Therefore, stable transformants were generated, but at a lower frequency than with the non-restrictive *S. lividans*. This theory is supported by MacNeil (1988) who found that the amount of restriction in *S. avermitilis* Ikeda was correlated with the number of potential restriction endonuclease sites in the plasmid DNA. The fact that the *S. scabies* strains were not transformed with any of the plasmids, likely indicates the presence of one or a number of restriction systems with broader specificity than that in *S. coelicolor* (Matsushima and Baltz, 1996).

For the purpose of calculating transformation efficiency, all of the protoplasts in the 50 µL aliquots were assumed to be viable. Based on SDS plating experiments, less than 0.1% of the material used was non-protoplasted mycelia. This value was low enough to be considered insignificant, so the efficiency was determined using the total number of protoplasts (1×10^{10}) in each 50 µL aliquot. Even though all of the protoplasts were considered viable in each of the experiments, it is also important to consider the regeneration potential of each species. The results in Table 2-4 show that regeneration of *S. lividans* was significantly ($p=0.05$) greater than the other three species. This higher regeneration potential could be used to explain some of the higher transformation frequencies also obtained with this species.

Passage of a plasmid through a non-restricting *Streptomyces* host, such as *S. lividans* has been shown to improve the efficiency of transformation into the restricting species (Fouces *et al.*, 2000). Other studies have found no improvement when this preliminary step was performed. Bailey and Winstanley (1986) attempted to introduce pIJ702 into the restrictive species *S. clavuligerus* Higgens and Kastner after first passing it through the non-restrictive *S. lividans*, but obtained few transformants. Successful transformations occurred when the plasmids were isolated from previously transformed *S. clavuligerus*, or the protoplasts were subjected to heat treatment before transformation to inactivate the restriction system.

In the present study, the plasmid pIJ486 was maintained in *S. lividans* TK64 before its use in electroporation and transformation. However, *S. scabies* transformants could not be generated using this plasmid, supporting the results of Bailey and Winstanley (1986). It is likely that this plasmid was introduced into *S. lividans* more efficiently than into *S. coelicolor* (Table 2-10) partly because of being housed in *S. lividans* prior to transformation. The success of *S. coelicolor* transformations and electrotransformations with pIJ486 (Tables 2-9 and 2-10, respectively) again illustrates the potential differences between the restriction systems of this species and *S. scabies*.

Methylating strains of *E. coli* were used to house pHJ400 and pJOE2577 while plasmids pIJ486 and *Oaac* were housed in non-methylating strains. All four plasmids were introduced into the restrictive *S. coelicolor* with (pJOE2577 and pHJ400 from the methylating hosts being introduced at the lowest frequency (Table 2-9). These results indicate that *S. coelicolor* likely possesses a methyl-specific restriction system. However Table 2-9 also indicates a lower frequency of introduction into *S. coelicolor* than *S. lividans* of those plasmids (pIJ486 and *Oaac*) not maintained in methyl-proficient hosts. Therefore, there could also be a classic restriction system that recognizes and degrades non-methylated foreign DNA.

The results of Bailey and Winstanley (1986) suggest that *S. clavuligerus* possesses both a restriction system and a modification system that would allow DNA to avoid the restriction barrier. It would be interesting to use the plasmids introduced into *S. scabies* through conjugation for transformation of other *S. scabies* cultures to determine if efficiency could be improved. Successful transformation with such a plasmid would provide corroborative evidence of a restriction system and a modification system, although the two systems would require further characterization. For example, identification of individual restriction endonucleases from active fractions of disrupted *S. scabies* mycelium and characterization of their cleavage sites (Nomura *et al.*, 1995), and the use of actinophages as bacterial indicators (Cox and Baltz, 1984) would aid in identification of potential restriction system(s) in *S. scabies*.

Altering the timing of soft agar overlay was implemented to try to increase transformation frequencies. The timing was varied from 27 to 48 h, depending on when the first signs of growth were evident on the regeneration plates. The specific time of overlay did not affect the transformation frequency, as long as the colonies were visible. Plates that were overlaid before the first colonies appeared, even at 48 h, failed to generate any transformants. Plasmid borne antibiotic resistance gene expression is not related to normal bacterial secondary metabolism. It may be that exposure to the antibiotic is required for expression of resistance. Therefore, survival is not linked to growth phase but is likely determined by the number of colonies existing at the time of overlay. If the overlay is applied too soon there will simply not be enough cells present to survive the initial antibiotic exposure. However, if the overlay is not added until a confluent lawn of cells has grown, there is the potential that some non-transformed colonies would not be exposed to the antibiotic and therefore survive. There is also the possibility of satellite colonies being protected by resistance in nearby transformed colonies.

2.4.1.1 Effects of Vectors

There was a significantly ($p=0.05$) lower frequency of transformation of pJOE2577 than with the other vectors, for *S. lividans* and *S. coelicolor* (Table 2-9). The delivery plasmid pSG5 on which pJOE2577 is based, has a relatively low frequency of transfer for *Streptomyces* spp. (Kieser *et al.*, 2000) and therefore a reduction in the number of pJOE2577 transformants was obtained. It is also probable that the elevated temperature of 40°C, required to cure the delivery plasmid, is slightly inhibitory to growth of bacterial species that normally grow at 30°C. As a result, fewer stable transformants were generated. Replication of the naturally temperature-sensitive delivery plasmid pSG5 stops above 34°C (Muth *et al.*, 1995), leading to a gradual loss of the plasmid. It may be of interest to see if temperatures of 35-39°C, which allow better sporulation and growth of the host *Streptomyces* spp., are sufficient to cure the plasmid. If these temperatures are practical, it is likely that higher transformation efficiencies would be achieved. The significant (p=0.05) difference in transformation frequencies between *S. lividans* and *S. coelicolor* is most likely due to the inhibitory presence of the *S. coelicolor* modification/restriction system, as indicated previously. However, lower frequencies could also result from the decreased ability of *S. coelicolor* to sporulate at 40°C, when compared to *S. lividans*.

The Tn*5493* transposon is a derivative of the composite transposon Tn*5*. Transposition of Tn*5* is very efficient in *S. lividans* (Kieser *et al.*, 2000). To further enhance transposition, two mutations were introduced into the *tnpA* gene used in pJOE2577. The reported transposition frequencies of Tn*5493* were determined to be consistent with the frequencies of transformation obtained in this study (Volff and Altenbucner, 1997b; Herron *et al.*, 1999).

Transposons are the tool of choice for mutagenesis because of their ability to randomly integrate into the host genome. However, the *S. lividans* and *S. coelicolor* transformants analyzed in this study show some site specificity with the pJOE2577 integrative transposon (Figures 2-4). Some researchers believe that this is the result of sibling selection, rather than integration at a preferred site (Smith and Dyson, 1995; Volff and Altenbuchner, 1997). Although this is a possibility, putative transformants were serially streaked to individual plates 3-4 times prior to selection and analysis to ensure that they arose from an individual spore.

Smith and Dyson (1995) also believe that the common integration site could be the result of a site-specific recombination event that occurred at a low frequency. However, when untransformed cultures of the *Streptomyces* species were probed with the Tn*5493* transposon or the entire pJOE2577 vector, no hybridization occurred (data not shown). These results indicate insufficient similarity between the native genome and pJOE2577 sequence for a site-specific recombination event to occur.

The final explanation for identical insertions is that there is some level of site preference for integration of the transposon. Southern hybridization and pulse field gel electrophoresis (PFGE) studies demonstrated that other transposons have a slight tendency to transpose into a specific site (Henderson *et al.*, 1990; Smith and Dyson, 1995; McHenney *et al.*, 1998; Gehring *et al.*, 2000). Anderson (1995) believes that the frequency of transposon insertion is not only dependent on the element used, but is also highly gene-dependent. Some genes are good targets for transposon insertion, and others are not. An accepted explanation for this phenomenon has not yet been proposed.

2.4.2 Electroporation

Electroporation was successfully used to introduce pIJ486, pHJL400, and *Oaac* vectors into *S. lividans* and *S. coelicolor*. In contrast to PEG-assisted transformation, neither species was effectively electrotransformed with pJOE2577. For all plasmids tested, the efficiency of electrotransformation was significantly (p=0.05) lower than that for traditional transformation. Even with lysozyme pre-treatment of the mycelia to facilitate pore formation, DNA uptake was inefficient.

The lysozyme treatment may not have been sufficient to weaken the mycelial walls, thereby impeding pore formation. Alternatively, if the incubation time was too long it may have led to protoplast formation and poor regeneration. Even though the concentration of lysozyme used in preparation of electro-competent mycelia was 10-fold less than that used in protoplast formation, lysozyme incubation conditions vary with species, as determined in this study. As a result, the level of lysozyme may have been enough to initiate protoplasting and contribute to the low transformation efficiency observed. Microscopic analysis of the mycelia during lysozyme treatment may show the degree of protoplasting.

Only pre-treated mycelia were used for electroporation in this study. There is no evidence in the literature indicating whether *S. lividans*, *S. coelicolor*, and *S. scabies* fragment during growth. However, judging by their tendency to clump during growth, and from cursory microscopic analysis of the mycelia grown in liquid culture, none of these species seem to exhibit fragmentation. If this technique is to be used in the future, attempts should be made to optimize the conditions for sonication of the mycelia prior to electroporation. The demonstrated low efficiency of electroporation of *S. lividans* (Pigac and Schrempf, 1995; this study), which is an easily transformable species, suggests other methods for DNA introduction, such as transformation and conjugation are better choices.

2.4.3 Conjugation

Conjugations were successful at introducing DNA into all of the *Streptomyces* spp. used in this study, in a vector-specific manner. The success of conjugation stems from its potential to shield the incoming plasmids from the restriction systems. During conjugation, contact between the donor and host cells limits exposure of the foreign vector to the surrounding environment, where nuclease attack commonly takes place. In addition, conjugation involves the transfer of single-stranded (ss) DNA, which is generally refractory to restriction endonuclease attack, although other studies indicate that the methyl-specific restriction systems cannot be evaded by the use of ss DNA (Oh and Chater, 1997).

The conjugal matings between the *E. coli* host and the mycelial preparations of *S. scabies*, *S. lividans*, and *S. coelicolor* were more successful than matings using spores of the same species as recipients. It is possible that this is simply due to the amount of conjugative material used, as there was more mycelium than spores available in each conjugation. The excess of mycelium allowed a larger number of introduced plasmids to avoid restriction and form stable exconjugants. Typically, 10^8 spores were used for each conjugation (Mazodier *et al.*, 1989; Flett *et al.*, 1997), however, this study used 10^9-10^{10} spores per reaction. Conjugations with *S. scabies* that utilize a larger number of spores than were used in this study may identify the threshold spore number required to generate a number of exconjugants equal to the number achieved using mycelium.

Although the lack of a sufficient number of spores may be one reason why mycelial conjugations were more effective than spore conjugations, there is also a possibility that the spores were not exposed to a temperature that would promote adequate pre-germination. Spores are thick-walled, pigment-protected survival structures, which are naturally resistant to environmental pressures. As a result, pre-germination by exposure to elevated temperature was required to promote uptake of foreign genetic material. This study determined that the recommended temperature of 50°C (Kieser *et al.*, 2000) was too extreme for *S. scabies* spores. Therefore, a lower temperature of 40°C for 10 min was selected to pre-germinate the spores. This lower temperature may not have been sufficient to initiate germination in the majority of spores. Ungerminated spores would remain resistant to the foreign DNA and only the few germinated spores would generate exconjugants. This could account for the lower frequency of exconjugants obtained when spores were used as recipient material, as opposed to mycelium.

If spore conjugations are used in future experiments, the heat-treated spores should be visualized microscopically to ensure that the temperature and length of incubation are appropriate to allow sufficient germination. A mating system that efficiently uses unigenomic spores as recipients of foreign DNA is particularly attractive for introduction of integrating vectors. The multicellular nature of mycelia makes identification of individual cells that are true transconjugants more difficult. Conjugation introduces an integrating vector into the genome in a completely random manner, resulting in one copy, multiple copies, or no copies per cell. In addition, the physical structure of the adjoined mycelial cells may allow selection of some false positives if they are near a cell containing the vector. In this study, each of the potential exconjugants were selected after multiple transfers to separate segregating colony types and identify the true transconjugants. If the vector was introduced into the single genome of a spore, decisive selection would be greatly enhanced.

After the exconjugants were purified, they were grown up in liquid media as indicated previously. Nalidixic acid was added to selectively inhibit the growth of *E. coli* while streptomycetes are naturally resistant. Cell division and DNA synthesis in *E. coli* are inhibited by nalidixic acid (Latch and Margolin, 1997), resulting in elongated filaments that are many times the length of normally dividing cells. This selection ensured that the plasmids were isolated from exconjugants and not from lysed *E. coli* cells that were carried over from the initial reaction. Despite the addition of nalidixic acid, some liquid cultures, particularly *S. lividans*, were contaminated by *E. coli*. Contamination was eliminated by increasing the concentration of nalidixic acid from 25 µg/mL to 50 µg/mL in liquid culture.

2.4.3.1 Effect of Plasmids

The integrative plasmid pSET152 was successfully introduced into all three *Streptomyces* spp. by conjugation (Figure 2-1). This is the first known demonstration of conjugal DNA transfer into *S. scabies* through conjugation, or any other method. Although the number of exconjugants was significantly ($p=0.05$) lower for *S. scabies* than for *S. lividans* and *S. coelicolor*, it was still sufficient to be useful for genetic analysis. Southern hybridization of putative exconjugants to a pSET152 probe (Figure 2-1) indicates site-specific integration of the vector in all of the species (Flett *et al.*, 1997). This is beneficial as there is no risk of potential gene inactivation events that may arise with random integration.

The reasons behind the success of plasmid pSET152 introduction into *S. scabies* over the other conjugative vectors remains ambiguous. Both pSET152 and pKC1218 contain the 760 bp *ori*T fragment from the IncP plasmid RK2 (Bierman *et al.*, 1992). In addition, they both possess *E. coli* replication functions from pUC19 (Kieser *et al.*, 2000). Plasmid pHJ401 also contains the pUC19 replicon for replication in *E. coli* (Larson and Hershberger, 1986). The major difference between the plasmids is that pKC1218 and pHJ401 contain a portion of the *Streptomyces* replicative functions (rep$^{SCP2^*}$). Even though the SCP2* replicon is mutated to allow increased stability, the plasmids do not contain the partition function required for the replicon to be functional. In addition to the absence of replication factors, pKC1218 and pHJ401 plasmids are also deficient in SCP2 stability factors. Because of these characteristics, they are easily lost from the host cell. After one round of sporulation, = 50% of the plasmids will be lost without selection (Kieser *et al.*, 2000). Low stability and no replication are desirable when the plasmid is used as a shuttle plasmid, delivering homologous segments of DNA. However, exconjugants formed only by these autonomously replicating plasmids would not be as stable.

The plasmid pSET152 is also unable to autonomously replicate in its *Streptomyces* host, and specifically integrates into the genome via the F C31 attachment site. This allows the formation of stable exconjugants that are propagated without a detectable loss of plasmid marker. It is probable that the extra stability obtained upon pSET152 insertion into the genome is required for generation of *S. scabies* exconjugants. There is the possibility that exconjugants containing pKC1218 and pHJ401 were formed, but not stably maintained, and the plasmids were lost before exconjugants could be identified. Also, vectors with the capability of site-specific integration are taken up much more efficiently than those that rely on recombination (Lillehaug *et al.*, 1997).

The generation of exconjugants when *S. coelicolor* and *S. lividans* were used as recipients indicates that the constructions of pKC1218 and pHJ401 are conducive for stable introduction into some species of *Streptomyces*. The first conjugations reported in the literature utilized the non-restrictive species *S. lividans* (Mazodier *et al.*, 1989). The lack of restriction systems in this species allows the recipient to easily take up foreign DNA. Even though these exconjugants would have been affected by the low stability, just as the S. *scabies* exconjugants were, more exconjugants were formed because more plasmids escaped restriction. As a result, the effect of the loss of a percentage of plasmids was not as noticeable on exconjugants numbers. Because conjugation with S. *scabies* is not as efficient as that with other *Streptomyces* spp., any loss of plasmids would significantly impact the number of exconjugants obtained.

There is evidence that some restriction systems have specific sites for restriction recognition (Murray, 2002). Therefore, the limitation in generating *S. scabies* exconjugants with all three of the vectors most likely lies in the ability of the *S. scabies* restriction system to target specific sites in particular vectors. Efficient conjugal transfer allows a sufficient number of plasmids to evade degradation so that stable exconjugants are generated. Results obtained from the transformation and electroporation experiments suggest that the restriction system in *S. scabies* is different from that in *S. coelicolor*. The success of electroporation and transformation using *S. coelicolor*, and the failure of the same conditions to introduce DNA into *S. scabies* highlight the differences of the two restriction systems. However, it is possible that both species posses both methyl specific and classical restriction systems. Possibly, the type of restriction system(s) in *S. scabies* requires a higher number of successful conjugations before plasmids are able to escape degradation.

From these results it can be proposed that the plasmid pSET152 was stably integrated into the chromosome of all three *Streptomyces* species and was not lost upon replication. Its construction allowed a sufficient number of plasmids to successfully circumvent the *S. scabies* restriction system, while the majority of the other plasmids were recognized by the system and degraded.

2.4.4 Future Work

The future potential of pSET152 as a delivery system for *S. scabies* should be explored. The integrative pSET152 may be useful in mutagenesis experiments if the insert is a transposon that could be released from the delivery vector and integrate elsewhere in the genome. However, integration at the *att* site in the genome may occur too rapidly to allow for transposition into a random site in the genome. Analyses were initiated by forming a pSET152::Tn*5493* ligation product and introducing it into the three species of *Streptomyces* through conjugation (data not shown). Potential exconjugants were generated from *S. lividans* and *S. coelicolor*, but not from *S. scabies*. Transfer of the transposon Tn*5493* through traditional transformation was unsuccessful in *S.*

scabies. It is possible that the natural restriction system recognized and degraded Tn*5493* before integration took place. Work in this area should allow identification of a vector/transposon that could be introduced into *S. scabies* via the pSET152 vector, in order to acclimatize it to the restriction systems that are present. The isolated vector may than be used to re-transform *S. scabies*, either by conjugation or traditional PEG transformation, at high enough frequencies to be useful in genetic analysis.

Future experiments should also proceed to determine the utility of pSET152 as a vector for complementation studies using mutagenized homologous DNA inserts for specific gene inactivation or for increasing copy number of a particular gene to determine phenotypic effects. Utilizing a synthesized DNA insert such as a PCR product, as opposed to the cloned fragment, is another method to assess the ability of pSET152 to function as a delivery plasmid in complementation studies. The PCR product would not be methylated and could potentially be constructed so as not to be recognized by the native restriction systems. Integration of pSET152 based vectors occurs at the neutral *attB* site within the host genome, therefore insertion of this vector will no have a phenotypic effect, except that intended by the accompanying DNA fragment. If this fragment is large, homologous recombination is possible (Matsushima *et al.*, 1994). It may also be possible to use pSET152 as a delivery plasmid to introduce a particular vector into *S. scabies*, so it becomes acclimatized to the specific modification system(s) present. After the plasmid is isolated and separated from pSET152, it should be transformable into *S. scabies* at usable frequencies.

Although transposon systems have been developed for use in *Streptomyces*, none have been successful in generating a large number of random insertions (Gehring *et al.*, 2000). Furthermore, in only two cases were the insertional mutations linked to the resulting phenotypic changes (Ikeda *et al.*, 1993; McHenney *et al.*, 1998). With our current level of technology and the size of the *Streptomyces* genome, this mode of investigation within *Streptomyces* is limited. One possible alternative is to create a genomic library that will be the recipient of the insertional mutation vectors in *E. coli* or *S. lividans*, as was done by Gehring *et al.* (2000). This could help alleviate both the negative influence of the restriction system and the size of the genome that needs to be screened. Recently the complete sequence of *S. coelicolor* A3(2) was determined (Bentley *et al.*, 2002). Despite the heterogeneity between *Streptomyces* spp., this information will be of immense value in genetic analysis of the other species, including *S. scabies*. The combination of the complete genome sequence and new techniques such as DNA microarrays will also make mass screening of the unknown *S. scabies* genome more feasible.

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Chapter 3 Hybridization of the *hrp* Gene Cluster from *Pseudomonas syringae* pv. *phaseolicola* to *Streptomyces scabies*

3.1 Introduction

The ability of phytopathogenic bacteria to produce the hypersensitive response (HR) in plants is, for the most part, directed by the bacterial Hrp (hypersensitive response and pathogenicity) system (Lindgren, 1997). The *hrp* genes are localized in clusters of 20-25 kb and have been found in all major plant pathogenic bacteria including *Erwinia amylovora* Burrill, *Erwinia chrysanthemi* Buckholder, McFadden & Dimock, *Pseudomonas syringae* Burkholder, *and Xanthomonas* spp. Doidge. (Bonas and Van den Ackerveken, 1999). To date there have been no reports of this system in Gram-positive bacteria.

The ability to elicit the hypersensitive response in susceptible plant hosts is an easily identified marker of pathogenicity, and as such, study of the relatively simple hrp/HR system is considered a means by which to elucidate the specific aspects of pathogenicity. Therefore, understanding the Hrp system has become an important focal point of bacterial research.

One of the most important protein products secreted when the Hrp system is expressed is harpin. Harpins are glycine-rich proteins that lack cysteine and that don't contain an amino terminal secretion signal, normally associated with secreted proteins. Instead, the signal is encoded within the first 15 codons of the mRNA. Proteins without amino terminal signals are secreted through type III secretion systems. Although it is understood that the secreted protein product harpin is involved in the HR of plants to pathogen invasion, the actual function in this system is not yet known (Bauer *et al.*, 1995).

There have been three harpins identified so far, homologs of all three may be found in any given pathogenicity system. Of the three harpins, HrpN was the first to be isolated and characterized. It was discovered when transposon mutants were no longer able to produce HrpN, induce HR, or cause disease (Wei *et al.*, 1992). Of the other two harpins HrpZ and HrpW, the HrpZ protein is found in association with the cell wall of plants and with the newly formed bacterial pilus (Brown *et al.*, 2001). This suggests that it may function in delivery of the avirulence gene products. Recently Lee *et al.* (2001) showed that HrpZ integrates into plant lipid membranes and forms an *in vitro* ion-conducting pore that is believed to be involved in nutrient release from the host cell and/or delivery of avirulence factors to the plant. The HrpW protein is only loosely considered a harpin as it possesses the same amino-terminal domain of the harpins, but has a carboxyl-terminal pectate-lyase like domain. As a result, it is proposed that HrpW interacts with pectic fractions in plant cell walls.

The pilus is another important component of the Hrp system. Encoded by *hrpA*, the pilus forms on the surface of the pathogenic bacteria (Roine *et al.*, 1997). This is reinforced by the fact that *hrpA* mutants are unable to initiate the HR response and cause disease (Anderson and Schneewind, 1999). Although its role in disease initiation has not yet been identified, it is hypothesized that the pilus permits injection of the Hrp proteins directly into plant tissues. Through immunogold labelling of HrpA, Brown *et al.* (2001) were able to visualize the Hrp pilus penetrating, and most likely crossing, plant cell walls that were adjacent to the invading bacteria.

The *hrp* gene cluster also contains genes involved in the type III secretion system (Gabriel, 1999). Once harpin and the other bacterial Hrp proteins are produced, they must be secreted across the bacterial membranes for direct interaction with the host plant. Hrp-dependent translocation of avirulence proteins into plant cells has been difficult to verify. However, Mudgett and Staskawicz (1999) have recently provided evidence for translocation of *P. syringae*-derived AvrRpt2 protein to the *Arabidopsis* host cell during infection. Rossier *et al.* (1999) have also shown a relationship between the secretion of Avr proteins and the Hrp system, in *Xanthamonas campestris* pv. vesicatoria Doidge. The Hrp gene products implicated in secretion include HrpU and HrpC, which assist in translocation of HrpZ across the bacterial inner and outer membrane, respectively (Huang *et al.*, 1995; Charkowski *et al.*, 1997). There is also involvement of HrpR, S, T, and E, although their roles must still be clarified.

Like all systems, the Hrp system requires internal regulation to ensure optimum activity. HrpB activates expression of various transcriptional units (Rossier *et al.*, 2000); HrpR, S, and L positively regulate/activate expression of *hrp* and *avr* genes (Grimm *et al.*, 1995); HrpK is a conserved effector; HrpV is required for HrpN secretion and also functions as a repressor of *hrp* gene expression; and HrpG is believed to be the first protein in the regulatory cascade as expression of all other *hrp* genes is eliminated in HrpG$^-$ mutants (Wengelnik *et al.*, 1996).

Although there are similarities between the Hrp systems in different bacterial strains, there are also differences in specific regulation and activation of the genes. There have been two groups of Hrp systems identified based on organization of *hrp* genes, DNA sequences, and *hrp* regulatory systems (Alfano and Collmer, 1996). The first group is found in *P. syringae* sp. (leaf spot of bean, tomato and *Arabidopsis*) and *E.*

amylovora (fire blight of apple and pear). The second hrp system is found in bacterial strains such as *X. campestris* (bacterial spot of pepper and tomato) and *Ralstonia solanacearum* Yabuuchi (bacterial wilt of tomato and potato). As expected, the Hrp systems of the first group are more closely related to each other than to those of the second group. There are nine *hrc* (hypersensitive and conserved) genes and two *hrp* genes that are conserved between the two groups (Bogdanove *et al*., 1996). However, between the two groups, the majority of *hrp* genes are completely different in sequence, arrangement and regulation.

Through sequence analysis Sawada *et al*. (1999) found that the genes *hrpL* and *hrpS* of the *hrp* gene cluster are stable on the chromosome of *P. syringae* and have never undergone horizontal gene transfer between the groups of *P. syringae*. They chose these genes, from opposite ends of the cluster, to be representative of the entire Hrp complex. This evidence may indicate that the *hrp* cluster is not as mobile as other environmentally selected traits such as antibiotic resistance (Egan *et al*., 1998), or other pathogenicity related factors. The incidence of homology between the *hrp* clusters of related pathovars of *P. syringae* (Willis *et al*., 1991) can be explained by conservation through evolution, rather than horizontal transfer. If the *hrp* cluster is not mobile within an individual bacterial strain, the occurrence of the same or highly homologous cluster in another strain such as *Streptomyces* is less likely.

However, others believe that because the Gram-negative bacteria in which the Hrp system has been identified are phylogenetically diverse and are widely distributed, there is still evidence of horizontal gene transfer (Alfano and Collmer, 1997). The influence of horizontal gene transfer on the occurrence of pathogenicity islands (PAIS) is recognized. The PAIS are large chromosomal clusters of closely related genes that include many virulence genes and are selectively present in pathogenic strains. They have different G+C content when compared to the native genome, are often flanked by inverted repeats, are bordered by mobile genetic elements and are highly unstable (Alfano *et al*., 2000). Based on these criteria, Healy *et al*. (1999) believe the locus ORF*tnp-nec1*-IS*1629* resides at the beginning of an as yet uncharacterized pathogenicity island, and has been transferred in a unidirectional manner to unrelated *Streptomyces* species, that is from *S. scabies* to *S. acidiscabies* and *S. turgidiscabies*. Because PAIS encompass genes involved in pathogenicity, it is logical to postulate that additional pathogenic factors like the Hrp system will be found within this particular island.

It is believed that *hrp* genes were introduced to *P. syringae* from an unknown enteric animal pathogen by horizontal gene transfer (Brown *et al.*, 1998). There is already convincing evidence of commonality between animal and plant pathogenesis mechanisms (Rahme *et al.*, 2000). For example, the type III secretion system is recognized as the main mode for export of the pathogenicity factor Yop in *Yersinia,* Sips and Sops in *Salmonella*, and Esps in enteropathogenic *E. coli* (Jackson *et al.*, 1999). In addition, type III secretion genes found in animal pathogens have been shown to reside in PAIS (Huek, 1998; Winstanley and Hart, 2001). Based on this evidence and the previous studies of bacterial phytopathogenic systems, the presence of plant pathogen *hrp* clusters within pathogenicity islands is a reasonable assumption.

This study was undertaken to determine if the Hrp cluster in *P. syringae* pv. *phaseolicola*, or a homologous sequence with the same function, could be identified in *S. scabies*. The evidence indicates that a large number of genes involved in pathogenicity are present because of horizontal gene transfer of pathogenicity islands (Gabriel, 1999; Healy *et al.*, 1999; Jackson *et al.*, 1999). Some of the strongest evidence in support of horizontal transfer is that the %GC content within these loci is radically different from the rest of the genome. Because of the high G+C content (73%) of *Streptomyces* there is a strong possibility that certain regions exhibiting low G+C content are not native, but rather have moved into the genome via horizontal transfer (Wright and Bibb, 1992; Healy *et al.*, 1999). The average G+C content of the *hrp* genes is around 58% (Alfano *et al.*, 2000), which would be distinguishable from the native *Streptomyces* genome.

The *hrp* genes responsible for producing harpin and encoding the type III secretion system, may have homologous counterparts in *S. scabies*. *Streptomyces* have been shown to utilize the type III secretion system to secrete antibiotics and other secondary metabolites such as thaxtomin A, a major pathogenicity factor of *S. scabies* (Lawrence *et al.*, 1990). In addition the characteristic scab lesions are zones of dead tuber tissue, supporting the possibility that harpin or a similarly acting component may be a common effector protein inducing an HR in the infected tuber.

To determine if *P. syringae* pv. *phaseolicola hrp* genes were present, *S. scabies* genomic DNA blots were hybridized to probes constructed from *P. syringae* pv. *phaseolicola hrp* sequences. The *hrp* cluster of *P. syringae* pv. *phaseolicola* spans two separate linkage groups. The first consists of the genes *hrpL, hrpAB, hrpC, hrpD, hrpE, hrpF, and hrpSR* and is approximately 22 kb in size, while the second is 3.7 kb and contains only *hrpM* (Scholz *et al.*, 1994). Previously, plasmid pPL11 was constructed from plasmid pWB5a and a 19 kb *Bam*HI-*Hin*dIII fragment of the *hrpA-S* linkage group (Lindgren *et al.*, 1989). Hybridization studies using this region as a probe against other *P. syringae* pathovars has shown homology (Lindgren *et al.*, 1988, Scholz *et al.*, 1994). The *hrp* fragments in plasmid pPL11 were used to probe the *S. scabies* genome.

3.2 Materials and Methods

3.2.1 Bacterial Strains, Plasmids and Growth Conditions

Streptomyces species (*S. lividans* TK64, *S. scabies* Lpit10 and Lpit17) were grown in 25 mL of Yeast Extract-Malt Extract (YEME) liquid medium (Hopwood *et al.*, 1985) at 30°C for 30 h. The origin of each strain is indicated in Table 2-1. *S. lividans* was grown in a 250 mL flask baffled with a metal spring, to avoid the formation of large mycelial clumps (Kieser *et al.*, 2000).

Escherichia coli DH5a containing the *hrp* cluster of *P. syringae* pv. *phaseolicola* in the plasmid pPL11 (Lindgren *et al.*, 1989) was a kind gift from P. Lindgren (North Carolina State University, Raleigh, NC USA). The transformed *E. coli* strain was grown in Luria-Bertani (LB) liquid broth supplemented with tetracycline at 15 µg/mL (Sigma) at 37°C.

3.2.2 DNA Isolation and Manipulation

Pseudomonas syringae pv. *phaseolicola* genomic DNA was isolated following the CTAB method for isolation of bacterial genomic DNA (Wilson, 1994). All *Streptomyces* genomic DNA was extracted following the rapid small-scale isolation of *Streptomyces* total DNA procedure (Hopwood *et al.*, 1985). Plasmid DNA of pPL11 was isolated using the Qiagen mini-prep purification system (Qiagen, Valencia, CA) and digested simultaneously with *Bam*HI, *Hin*dIII and *Eco*RI. This generated a 15.5 kb (*A-E*) *Bam*HI-*Eco*RI fragment and a 4.5 kb (*F-S*) *Eco*RI-*Hin*dIII fragment. The two fragments were separated by electrophoresis through a 0.7% (w/v) UltraPure agarose gel (GibcoBRL, Gaithersburg, MD), excised, and freeze-squeeze-purified. Total genomic DNA was digested separately with *Eco*RI, *Bgl*II, *Pst*I, or *Pvu*II. These enzymes were chosen because their recognition sites are low in G+C content and they generated relatively large *Streptomyces* DNA fragments.

3.2.3 Southern Hybridization

The digested genomic DNA was separated through a 0.7% (w/v) UltraPure agarose gel (GibcoBRL) in 1X TAE buffer at 12 V for 18-20 h. The DNA was NaCl depurinated, transferred to N+ nylon membrane (Amersham Pharmacia, Buckinghamshire, England) with 0.4M NaOH, and crosslinked to the membrane using a UV Stratalinker (Stratagene, LaJolla, CA) according to manufacturer's instructions. Between 80-100 ng of the two *hrp* fragments, 15.5 kb (80 ng) and 4.5 kb (100 ng), were randomly labelled separately with [a-^{32}P]dATP using the Canadian Life Tech Kit (Canadian Life Technologies, Burlington, ON). Lambda DNA (50 ng) digested with *Hin*dIII DNA was also labelled as a size marker.

The membranes were pre-hybridized in pre/post hybridization buffer (1% w/v sarkosyl, 200 mM NaCl, 10 mM Tris-HCl (pH 7.5-8.0), 1 mM EDTA) for 30 min at 60°C and then in hybridization buffer (1% sarkosyl, 1 M NaCl, 50 mM Tris (pH 7.5), and 10% w/v dextran sulfate added at time of use) for 1 h at 60°C. The two probes were pooled and added to the hybridization buffer at a final concentration of 10^6 cpm/mL, and hybridized at 60°C for 16-18 h. The blot was washed twice in 2X SSC and 0.1% SDS at 60°C for 15 min. The membranes were exposed for 12 h to Fuji SuperRX film (Fuji, Tokyo Japan) using a Biomax intensifying screen (Eastman Kodak, Rochester NY) at –80°C.

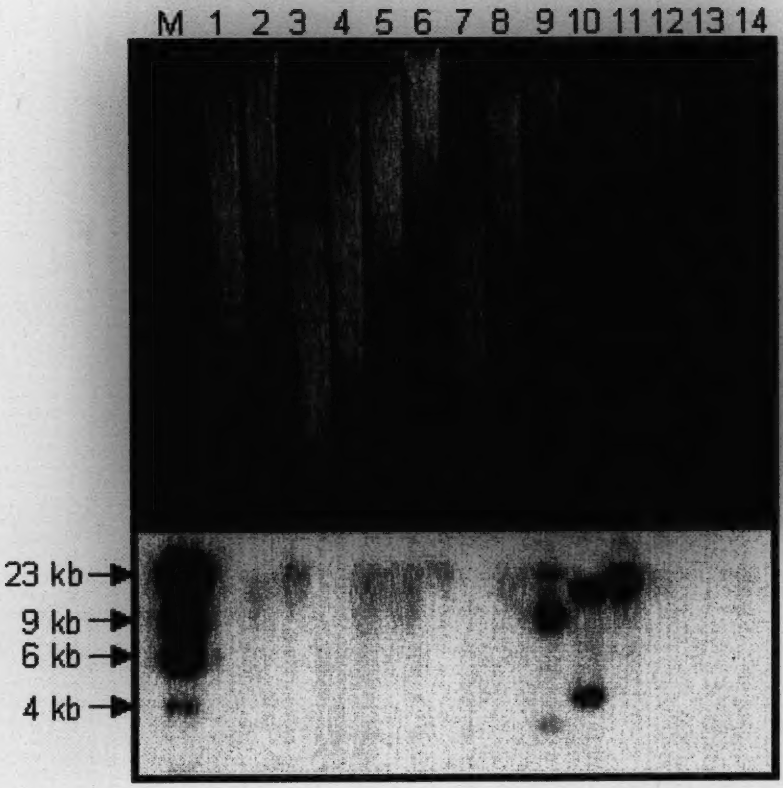
3.3 Results

Two fragments from plasmid pPL11 containing the *hrpA-E* and *hrpF-S* genes were pooled for use as probes in the same hybridization reaction. The autoradiogram obtained by hybridizing and washing under low stringency conditions shows no evidence of homology of the *P. syringae* pv. *phaseolicola hrp* cluster with *S. scabies* (Figure 3-1). Lanes 9-11 show three distinct banding patterns of *P. syringae* pv. *phaseolicola* when digested with *Bgl*II, *Pvu*II, and *Sst*II, respectively. However, no hybridization is visible in *S. scabies* or *S. lividans*. The background signal that is seen in the *S. scabies* strains, is non-specific hybridization that is expected with low stringency conditions and genomes that are high in G+C.

3.4 Discussion

In order for Southern analysis to be successful, there is a minimum homology required between the probe and prospective sequence. If this homology is less than

Figure 3-1. Agarose gel (top) of digested *Streptomyces* spp. and *Pseudomonas syringae* pv. *phaseolicola* genomic DNA. Lanes 1-4, *S. scabies* Lpit10 genomic DNA digested with *Bgl*II, *Eco*RI, *Pvu*II, and *Sst*II, respectively; lanes 5-8, *S. scabies* Lpit17 genomic DNA digested with the same enzymes, respectively; lanes 9-11, *P. syringae* pv. *phaseolicola* genomic DNA digested with *Bgl*II, *Eco*RI, and *Hin*dIII, respectively; lanes 12-14, *S. lividans* genomic DNA digested with *Bgl*II, *Pvu*II, and *Sst*II, respectively. The corresponding Southern autoradiogram (bottom) represents *Streptomyces* spp. and *Pseudomonas* genomic DNA probed with the 15.5 kb *Bam*HI-*Eco*RI (*hrpA-E*) and the 4.5 kb *Eco*RI- *Hin*dIII (*hrpF-S*) fragments from plasmid pPL11. Lanes are the same as in the agarose gel, with the addition of lane M containing ? *Hin*dIII-digested DNA. Size in Kb is indicated on the left side.


M
1
2
3
4
5
6
7
8
9
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11
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13
14
23 kb
9 kb
6 kb
4 kb

around 60% the chance of recognition, even under low stringency conditions diminishes. When Lindgren *et al.* (1988) used probes based on *P. syringae* pv. *phaseolicola* in Southern hybridization reactions with DNA from various *Xanthomonas* pathogens, no hybridization was observed. Even though the two groups contain functionally analogous pathogenicity genes, there are obviously sequential or organizational differences that have arisen during evolutionary divergence. This study reflects the results obtained by Lindgren *et al.* (1988), as no sequence with sufficient homology to the *P. syringae* pv. *phaseolicola hrp* cluster was found in *S. scabies.*

Two Hrp system groupings with mostly unique genetic organization and composition have been identified. There is some evidence that indicates the involvement of a group II Hrp system rather than a group I system in pathogenic *Streptomyces*. For instance, transcription of the *hrp* genes and growth of the bacteria *P. syringae* pv. *phaseolicola* and *E. amylovora* is seen at an acidic pH (5.5-5.7). However, for *X. campestris* pv. *vesicatoria*, *hrp* gene expression is almost abolished at acidic pH (Rossier *et al.*, 1999). Scab development induced by *S. scabies* on potato tubers is impeded at acidic pH. It would be interesting to test *S. acidiscabies*, which also causes potato scab and flourishes in acidic soil, for the presence of the *P. syringae hrp* gene cluster.

As mentioned previously, the Hrp cluster is involved in hypersensitivity and pathogenicity, both of which are related to virulence. Because *S. lividans* is not pathogenic there is a greater chance that this type of system is not represented in this species. One of the defining characteristics of pathogenicity islands is their presence in virulent strains of a species and their absence in non-pathogenic strains (Winstanley and Hart, 2001). If the Hrp cluster does reside in a PAI, as is expected, its absence from the non-pathogenic *S. lividans* would be anticipated.

Further work should concentrate on localization of the more highly conserved *hrc* genes, especially those from Group II, which is more closely related to Streptomycetes. Development of degenerate primers or probes constructed from the highly conserved regions between the two *hrp* groups, or a composite of *hrp* genes from a variety of different bacterial strains, may facilitate further investigation.

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Chapter 4 PCR Amplification of an *arpA* Homologue in *Streptomyces scabies*

4.1 Introduction

Single-celled bacteria are able to communicate with each other, and display multicellular activity. Only recently has the term quorum sensing been assigned to all of the processes that allow bacteria to monitor their behaviour and that of the group. Quorum sensing describes the ability of bacteria to determine their cell density within their constantly changing environment, by detecting the level of hormone- or pheromone-like chemicals that the individual cells secrete (Fuqua *et al.*, 1996, Robson *et al.*, 1997). When the concentration of these pheromones indicates that the group of bacteria is large enough to initiate expression of a particular phenotype, the group is known as a quorum. Quorum sensing may also be referred to as cell density-dependent gene expression.

Cell communication and coordination of activities is important in a number of different bacterial strategies. For instance, pathogenic bacteria must coordinate their attack at such a time that their numbers are sufficient to overwhelm the hosts' immune defences. At the critical density, all cells within the quorum are triggered to attack the host. While factors such as pH, osmoregularity, temperature and nutrient availability are responsible for environmental control of bacterial gene expression, quorum sensing may be the main factor responsible for regulation of virulence.

Research in bacterial quorum sensing began when cell density control of bioluminescence autoinduction was detected in the marine bacterium *Vibrio fischeri*, and its free-living relative *Vibrio harveyi* (Nealson *et al.*, 1970). More recently Salmond *et al.* (1995), while searching for genes involved in the antibiotic biosynthetic pathway of *Erwinia carotovora*, discovered that certain mutants could only make carbapenem antibiotics when grown in association with a different group of mutants. It was later discovered that the second mutant was supplying a signalling molecule, which was required to trigger the antibiotic synthesis in the first group. Characterization of the signalling molecule identified it as the same autoinducer used by the completely unrelated *V. fischeri* to generate bioluminescence. The discovery that two diverse organisms utilized the same signalling molecule led to the belief that cell communication based on chemical signalling was a common mechanism throughout the bacterial world.

Gram-negative bacteria have been the focus of the most extensive work in quorum sensing. Generally, N-acyl-homoserine lactones (AHLs) are the diffusible communication molecules, or autoinducers, used in regulating the cell density-dependent phenotypes of Gram-negative bacteria (Hartman and Wise, 1998). Acyl HL molecules comprise an acyl chain conjugated through an amide linkage to a homoserine lactone moiety (Fuqua and Greenberg, 1998). The various signalling molecules differ mainly in chain length, oxidation and saturation (Dong, 2001). The variability of the different signals is most likely in response to specifics of the different bacteria, their range of environments, and their individual communication needs. It has been shown that AHLs are used by over 30 different species of Gram-negative bacteria to control different cell-density related functions (Bassler, 1999). An extensive list of the various systems used in Gram-negative quorum sensing is detailed in Swift *et al.* (1996). This list includes such organisms as *Agrobacterium tumefaciens* Conn (conjugation), *Escherichia coli* Migula (cell division), *Pseudomonas aureofaciens* Kluyver (antibiotic synthesis), and *Rhizobium leguminosarum* Frank (nodulation). A more recent study showed that over 250 genes are affected by the AHL signalling system in *Pseudomonas aeruginosa* Migula (Whiteley *et al.*, 1999).

In Gram-positive bacteria, processes also exist that are involved in cell-density dependent regulation. Such processes include the development of genetic competence in *Bacillus subtilis* Cohn and *Streptococcus pheumoniae* Chester (Bassler, 1999), the virulence response in *Staphylococcus aureus* Rosenbach, the production of antimicrobial peptides by various lactic acid bacteria, as well as differentiation and antibiotic production in *Streptomyces* (Gray, 1997). However, the Gram-positive signalling systems do not use AHL signals; rather, these systems involve post-translational processing of secreted peptide pheromones that are recognized by cytoplasmic response regulator proteins (Kleerebezem, 1997). Various classes of autoregulators have been identified in Gram-positive bacteria; a number were found in *Streptomyces* spp. The family of γ-butyrolactone autoregulators that control morphological differentiation and secondary metabolism is one of the most common (Teplitski *et al.*, 2000).

Butyrolactone compounds are structurally similar to homoserine lactone molecules. Despite the similarities in function and structure, the biosynthetic pathways and regulatory mechanisms of the two systems are different. To date, 11 butyrolactone autoregulators comprising three distinct types have been chemically identified. The distinction between the different types is related to structural differences in the C-2 side chains (Kawachi *et al.*, 2000). Specific receptors for each class, A-factor type (Horinouchi and Beppu, 1992), IM-2 type (Ruengjitchatchawalya *et al.*, 1995), and virginiae butanolides (VB) type (Gräfe *et al.*, 1982), have been cloned and characterized from several different *Streptomyces* species (Waki *et al*., 1997). The DNA-binding domains of the receptors have homology to the TetR family of transcriptional repressors, and are specific for one particular butyrolactone. The presence of butyrolactone homologues and their receptors, in a number of different *Streptomyces* spp. suggests that a general hormonal regulatory system controls morphogenesis and antibiotic production in the genus (Onaka *et al*., 1998; Kitani *et al*., 2001).

The best-studied γ-butyrolactone, A-factor [2-(6'-methylheptanoyl)-3R-hydroxymethyl-4-butanolide], is essential for yellow pigment production, aerial mycelium formation, streptomycin production, and low-level streptomycin resistance in *S. griseus* (Horinouchi and Beppu, 1992). Gel mobility shift assays have shown that ArpA, the receptor protein for A-factor, loses its affinity for its DNA binding site when A-factor is present (Onaka and Horinouchi, 1997). Essentially, when ArpA is bound to the promoter region of certain key metabolic and morphogenesis genes it functions as a repressor of differentiation and streptomycin production. This inhibition occurs during early growth when the concentration of A-factor is low, and prevents the expression of the bound regulatory genes. As A-factor concentration increases, it alters the conformation of the ArpA dimer causing dissociation from the DNA, which subsequently de-represses gene expression. As a result, aerial mycelium differentiation and streptomycin production occur.

Analysis of site-directed mutations of ArpA has led to the identification of various residues that are essential for both DNA-binding and ligand-binding activity (Onaka *et al*., 1997; Sugiyama *et al*., 1998). These data indicate that ArpA consists of two functional domains, one for DNA-binding and one for ligand binding. Recently the specific DNA target of ArpA was identified as *adpA* (Ohnishi *et al*., 1999). The transcriptional activator generated when A-factor accumulates, induces the various downstream genes involved in streptomycin production, aerial mycelium formation and sporulation. By restoring activity in various A-factor-deficient mutant clones, other genes involved in cellular differentiation, such as *orf1590*, the *amfR-amfA-amfB* gene cluster (Kudo *et al*., 1995), *afsA,* and *sgaA* (Ando *et al*., 1997) have been identified. These results are leading to elucidation of the signalling pathway involved in A-factor regulation.

Another γ-butyrolactone system that controls morphological differentiation is found in *S. coelicolor*. Six different γ-butyrolactones have been found in this *Streptomyces* species (Kawabuci *et al*., 1997). Onaka *et al*. (1998) cloned two γ-butyrolactone receptor genes, *cprA* and *cprB*, from *S. coelicolor*. Through gene disruption it was determined that CprA functions as a positive regulator of antibiotic production and sporulation, while CprB functions as a negative regulator. Because of significant similarity in the amino acid sequences of the DNA binding domains of CprA and CprB, it is believed that these two receptors recognize the same operator site. The relative concentration of each protein determines whether secondary metabolism and morphogenesis are activated or repressed.

There is also a mixture of regulatory factors for virginiamycin production in *S. virginiae* that are similar to A-factor. Although virginiae butanolides (VBs), like A-factor, are involved in antibiotic production they have no effect on morphogenesis. Okamoto *et al.* (1995) were able to isolate, clone and express the gene *barA*, which encodes the VB binding protein in *S. virginiae*. The protein BarA is also a DNA binding transcriptional repressor that dissociates from its target DNA in the presence of VB (Kinosita *et al.*, 1997; Nakano *et al.*, 1998). More recently a regulatory protein also controlling VB synthesis, *barX* was found upstream of the *barA* gene (Kawachi *et al.*, 2000). Since BarA looses its DNA-binding activity in the absence of BarX, and this activity is restored with recombinant BarX, it is proposed that complete DNA binding requires BarA-BarX interaction or association. Two of the BarA repressed genes *varS* and *varR*, which are involved in virginiamycin resistance, have recently been found downstream from *barA* (Namwat *et al.*, 2001). Through these studies the complete picture of VB transcription and signalling is being elucidated.

The last major class of butyrolactone factors is the IM-2 [(2R,3R,1'R)-2(1'-hydroxybutyl)-3-(hydroxymethyl)butanolides] of *Streptomyces* sp. strain FRI-5. Like the other autoregulators, IM-2 triggers production of blue pigment and the nucleoside antibiotics showdomycin and minimycin (Kitani *et al.*, 2001).

Despite recent advances, biochemical analyses of the density-dependent proteins are currently underdeveloped. Although many properties of butyrolactone signalling are conserved in homologous systems, a high degree of specificity is also observed. This is especially prevalent in the strict ligand specificity of receptor proteins from each autoregulator type (Nakano *et al.*, 1998). Becker *et al.* (1997) screened various strains of *S. scabies* for production of homoserine lactones that are homologous to the *Agrobacterium* autoinducer. Of the four strains tested, none were found to produce this particular autoinducer. However, this study is not completely definitive as the strains tested may simply not produce the homoserine lactone under the assay conditions, or they may be releasing a signalling molecule that is not specific for the *Agrobacterium* system. These problems are similar to those encountered when trying to develop a complete understanding of the signalling process.

The purpose of this study was to determine if key components of the A-factor type butyrolactone system exist in *S. scabies*. Using degenerate primers, the A-factor receptor gene, *arpA*, was first cloned and characterized from *S. griseus*. These primers were constructed based on the amino acid sequences of the amino-terminus and a lysyl endopeptidase-generated fragment, identified during ArpA purification (Onaka *et al.*, 1995). The primers were then used in polymerase chain reaction (PCR) amplification of a 150 bp band from *S. griseus* and various other *Streptomyces* sp. Onaka *et al.* (1998) used this 150 bp fragment was used as a probe to identify two ArpA homologues in *S. coelicolor* A3(2). Alignment of the amino sequence of ArpA, its *S. coelicolor* homologues, and various other butyrolactone receptor proteins shows a high degree of homology in the amino-terminal region used in primer design. This high degree of homology suggests that if present, it may be possible to detect a homologous receptor in *S. scabies*. The same primers were used in this study with *S. scabies* genomic DNA as the template.

4.2 Materials and Methods

4.2.1 Bacterial Strains and Growth Conditions

Spores of *S. scabies* (Lpit10 and Lpit17), *S. lividans* TK64, *S. coelicolor* J1501, and *S. clavuligerus* were used to inoculate 25 mL of yeast extract-malt extract medium (Kieser *et al.*, 2000). The cultures were grown at 30°C with vigorous shaking for approximately 30 h. The *S. lividans* culture was grown in a flask baffled with a metal spring, to prevent formation of large mycelial clumps.

4.2.2 DNA Isolation

Genomic DNA from each of the *Streptomyces* spp. was isolated as described in Hopwood *et al.* (1995) with the following modifications. Prior to addition of lysozyme the pelleted mycelium was freeze/thawed at –80°C/55°C, respectively, 2-3 times, to promote lysing. To facilitate lysing, the cultures were gently mixed with a transfer pipette after SDS was added. Transfer pipettes were also used to mix the phenol/chloroform into the samples, instead of vortexing suggested by the protocol. After the first precipitation with sodium acetate and isopropanol, the pellet was dissolved in 1X TE; no spermine-HCl was added before the second precipitation step was carried out.

4.2.3 PCR Amplification and Gel Electrophoresis

The PCR mix consisted of 30 mM KCl, 3.5 mM $MgCl_2$, 70 mM Tris-HCl (pH 8.0), 200 µM of each dNTP, 7.5% (w/v) DMSO, 0.1% (v/v) TritonX-100, 600 nM of each primer, 2 U platinum Taq polymerase (GibcoBRL, Gaithersburg, MD), and 1 µg genomic DNA template. The degenerate primers SEJ15 (5'-CCGAAGCTT-CGC-GCC-GT[C,G,A,T]-CA-[G,A]-AC-3') and SEJ16 (5'-GCCGAATTC-TT-GGA-GGC-GGA-GTG-[G,A]-AA-[G,A]-TA-3') were a gift from Susan Jensen, University of Alberta, and were designed as described previously (Onaka *et al.*, 1995). PCR reactions were performed with a hot start at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s. An extension step of 72°C for 5 min followed. A 12% DNA PAGE gel (40% acrylamide/0.4% bis-acrylamide) run at 60 V in 1X TBE running buffer was used to resolve 15 µL/lane of each PCR reaction. The PAGE gel was stained with ethidium bromide and visualized on a UV transilluminator.

4.3 Results

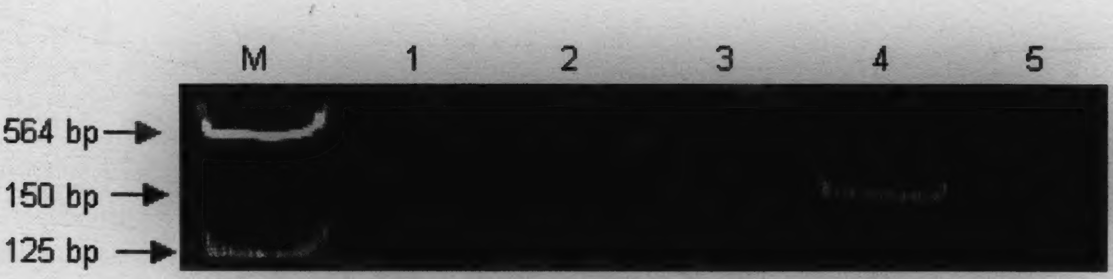
Under the PCR conditions used in this study, an *arpA* homologue was not amplified from *S. scabies.* The primers SEJ15 and SEJ16 should amplify a 150 bp fragment of the ArpA coding region, a product of the expected size is clearly evident in positive control *S. coelicolor* (Figure 4-1). There is also a faint band of the correct size detectable in *S. lividans.*

Several modifications of the PCR conditions with *S. scabies* were performed to optimize amplification. A range of $MgCl_2$ (0.5-2 mM), KCl (0-50 mM), template (5-250 ng/µL), primer (10-25 µM), and DMSO (0-10%) concentrations were used, as well as a range of annealing temperatures (30-46°C), and amplification on different thermocycler models (a Stratagene Robocycler with no ramp time and a Water-Cooled Ericomp with a ramp time). None of the modifications led to amplification of the 150 bp template DNA from *S. scabies.*

4.4 Discussion

Despite PCR-amplification of *arpA* homologues in a number of *Streptomyces* spp. under the conditions used in this study, an *arpA* homologue was not detected in

Figure 4-1. PCR amplification of *Streptomyces* genomic DNA template using the degenerate primers designed for the NH_2-teminal and an internal sequence of ArpA. The products were resolved on a 12% DNA-PAGE gel. Amplification of a 150 bp fragment from *S. coelicolor* (positive control) and *S. lividans* is shown. The lanes are as follows: 1, *S. scabies* Lpit10; 2, *S. scabies* Lpit17; 3, *S. clavuligerus*; 4, *S. coelicolor* J1501; 5, *S. lividans* TK64; M, *Hin*dIII–digested ? DNA. Fragment sizes in bp are indicated on the left.


M
1
2
3
4
5
564 bp
150 bp
125 bp

S. scabies. A distinct PCR product of the expected size was detected in *S. lividans* and *S. coelicolor*. The exact identity of this fragment was not confirmed through DNA sequence analyses.

Template DNA that is G-C rich typically has a higher melting temperature, which can increase the difficulty of achieving successful PCR amplification. The addition of compounds such as TritonX-100, DMSO, formamide and glycerol, as well as a high primary denaturation step, are proven to increase both specificity and yield of PCR product (Roux, 1995); the additives more reliably denature the DNA for subsequent amplification. Both TritonX-100 and DMSO were added to the PCR reactions in this study. No combination of concentrations resulted in amplification of the desired product. Although the use of cosolvents may lower the T_m of the template DNA, they may also affect *Taq* DNA polymerase activity and thermostability (Landre *et al*., 1995). If the enzyme is adversely affected there will be a general decline in amplification, which will negate any improvements in specificity. The PCR product sought was 150 bp, the minimum length for efficient amplification (Johnson, 2000). When these factors are combined, as they are in this reaction, it is not surprising that the PCR product proved difficult to amplify. This idea is supported as even amplification of the PCR product from *S. lividans*, which has been successful in the past, yielded limited product.

There are vast genetic differences between different *Streptomyces* species, thus PCR conditions favourable for amplification from one species will not necessarily be effective with another. Due to the similarity and close evolutionary development between *S. coelicolor* and *S. lividans*, it is not surprising that the same PCR conditions were successful in both strains (Takeuchi *et al*., 1996; Kreuze *et al*., 1999). *Streptomyces scabies* is evolutionarily distant from the former two species and, as such, is expected to be less genetically similar. Even though the conditions used in this study were successful for amplification of an *arpA* homologue from *S. lividans* and *S. coelicolor*, different parameters may be required for amplification from *S. scabies*. However, modifications of the general PCR conditions were made with no improvement in amplification from *S. scabies*.

Due to the evolutionary distance between *S. scabies* and *S. lividans*/*S. coelicolor*, the *S. scabies* butyrolactone system may possess a sequence that is too divergent from the A-factor type system to be identified with the primers used. The degenerate primers used increase the variability allowed in template amplification, however they have their limitations. Even a single base mismatch at the 3' terminus of the primer can prevent effective amplification (Johnson, 2000). Because *Streptomyces* spp. are genetically diverse, the chance of the primer recognition site including a variable region or position is great. As a result, the effectiveness of PCR to detect homologous sequences in different *Streptomyces* spp., including *S. scabies*, is negatively affected.

Future work in this area should begin with an assay to detect the presence of one of the various forms of exogenous butyrolactones in cultures of *S. scabies*. If found, the autoinducer could then be purified and used to identify the other components of the signalling system, in a manner similar to that used for characterization of ArpA.

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Chapter 5 General Discussion

Streptomyces play a vital role in numerous ecological and industrial processes. They are the source of a vast majority of the known antibiotics (Taddei *et al.*, 1999), and they are utilized in biocontrol of numerous pathogenic organisms (El Abyad *et al.*, 1993). Their native plasmids have been used to create many useful vectors for genetic research (Pernodet *et al.*, 1984; Smokvina *et al.*, 1990; Muth *et al.* 1995). The pathogenic *S. scabies* is responsible for common scab of potato, a significant disease found in all potato growing regions of the world. Unfortunately, very little information pertaining to the genetics of *S. scabies* has been acquired. As a result, any new information gleaned will significantly advance the state of knowledge of this important group of bacteria.

Genetic analysis of *Streptomyces* facilitates description of the structure of regulatory regions of the genome, elucidation of metabolic systems, study of secreted enzymes, and even an understanding of the restriction-modification systems that are the stumbling block in so many studies. A comprehensive knowledge of *Streptomyces* genetics is needed to fully exploit this genus' beneficial features. In terms of *S. scabies*, an increased understanding of the genetics and pathogenicity will lead to improved disease management and host resistance.

5.1 Status of *Streptomyces* spp. Genetic Analysis

Generally, there are three different routes followed in genetic analysis. The first identifies unknown genes, through insertional mutagenesis. The other two focus on identification of regions of homology between the known genes and sequences in the genotype under study, or by identification of a particular molecule (e.g. enzyme, antibiotic, etc.) and then subsequent analysis of the genes in its biosynthetic pathway.

This thesis concentrated on optimizing the search for both known and unknown genes. Transformation, conjugation and electroporation experiments were initially conducted to identify and characterize novel *S. scabies* genes. However, due to the resistance of *S. scabies* to genetic dissection, the focus changed to an analysis of the utility of these techniques with the various vectors. In the process, conjugation was optimized for *S. scabies* and a useful conjugative vector was identified. The process of looking for known genes was hampered by the specific properties of the *S. scabies* genome, as described below.

Taken together, the results of this thesis show the problems encountered when attempting genetic analysis of *Streptomyces* spp., especially with species that are particularly resistant to genetic manipulation, such as *S. scabies*. However, it also sheds some light on how such studies should proceed in the future.

The limitations of cloning by homology include a dependence on a sufficient level of homology between the probe/primer and the target DNA. In addition, only one gene may be sought at a time. This requirement of specificity was evident in the inability of both PCR amplification of *arpA* (Figure 4-1) and Southern hybridization of the *hrp* cluster to the *S. scabies* genome (Figure 3-1).

Even though the use of insertional mutagenesis in *Streptomyces* spp. is hampered, it has some potential in the search for specific genes. This technique has the advantage that a large number of gene interruptions, and subsequent phenotypic changes, may be generated.

The initial objective of this thesis was to identify genes from *S. scabies* that are involved in disease initiation or development. It is clear from the insertional mutagenesis, PCR amplification and Southern blot hybridization experiments that localization of these important genes is not a straightforward process. Genetic analysis is not only hindered by the recalcitrant nature of *S. scabies*, but each gene may possess different challenges in isolation, cloning and characterization.

5.2 Identification of Known Genes

This study focused on identification of genes within the *S. scabies* genome based on homology to known genes. The two genes were chosen for very specific reasons. The *hrp* genes, responsible for initiating the hypersensitive response, are ubiquitous throughout Gram-negative plant pathogens (Bonas and Van den Ackerveken, 1999). Secondly, they are commonly found in pathogenicity islands with a GC content that is significantly different from the surrounding genome. The difference in GC content is believed to indicate that these islands are routinely transferred among different bacterial genera. And finally, the *hrp* gene cluster contains genes involved in the type III secretion system (Gabriel, 1999). Secretion of antibiotics and other secondary metabolites by *Streptomyces* spp. has been shown to occur through the type III secretion (Lawrence *et al.*, 1990). For these reasons, it is plausible that the *hrp* cluster may be present in the pathogenic *S. scabies*.

The second gene sought in *S. scabies* was *arpA*, which encodes the receptor protein for A-factor γ-butyrolactone autoregulator. This system is involved in cell density dependent regulation. The control of morphological differentiation and secondary metabolism in *Streptomyces* is governed by cell-density dependent regulation (Teplitski *et al.*, 2000). Various classes of autoregulators have been identified in Gram-positive bacteria, a number of which were found in *Streptomyces* spp. The occurrence of *arpA* in a variety of *Streptomyces* spp. suggests the possibility that it may also be found in *S. scabies*.

Neither PCR amplification nor Southern hybridization were successful at identifying homologs of the *arpA* and *hrp* genes in *S. scabies*. Southern hybridization has been used previously to successfully identify *S. scabies* homologues of *txtAB* (Healy *et al.*, 2000). Amplification of genes by PCR has not been reported for *S. scabies*. As indicated before, both procedures are hindered by a requirement for significant homology between the sequence of interest and the probe/oligonucleotides primer used. The genetic diversity found in *Streptomyces* spp. complicates design of primers and probes with sufficient homology.

5.3 Identification of Novel Genes

This study demonstrated a methodical analysis of methods for DNA introduction into the plant pathogen *S. scabies*. Three different methods (conjugation, transformation and electroporation) were used to introduce different types of plasmids (integrating and autonomous vectors, and transposons carried on a suicide vector). Conjugation was determined to be the most amenable means for introduction of DNA into *S. scabies*.

Conjugal transfer of the integrative plasmid pSET152 into *S. scabies* was demonstrated (Figure 2-1). Transformation and electroporation were not successful for introducing foreign DNA into *S. scabies*. However, PEG-mediated transformation efficiently introduced all the vectors into *S. lividans* and *S. coelicolor* (Figures 2-2, 2-3, & 2-4). Electroporation facilitated plasmid introduction into both species, but at a significantly ($p=0.05$) lower frequency (Figures 2-2, 2-3, & 2-4).

The results suggest that DNA introduction into *S. scabies* is complicated by the presence of an uncharacterized restriction system. Identification of restriction systems in other species occurred because of a similar resistance to the acquisition of DNA from a different bacterial strain, or another bacterial species (Murray, 2002). The disparity of DNA introduction into *S. scabies* and the two control species, especially *S. coelicolor*, exemplify the differences in the restriction systems.

This is the first report of conjugal introduction of foreign DNA into *S. scabies*. The integrative plasmid pSET152 will be useful at introducing homologous sequences for mutation or gene dosage studies. The next step will be to ensure that the vector is able to function as a delivery plasmid.

5.4 References

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